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Fatty Acids, Fibre, Carotenoids and Tocopherols In Relation to Glucose Metabolism in Subjects at High Risk for Type 2 Diabetes

– a Cross-Sectional Analysis

Katriina Ylönen

Academic Dissertation

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Contents

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I to IV).

I Ylönen SK, Salminen I, Lyssenko V, Saloranta C, Aro A, Groop L, Virtanen SM, for the Botnia Study Group. Associations between fatty acids and glucose metabolism are affected by the Pro12Ala polymorphism of the PPAR-γ2 gene. Submitted.

II Ylönen SK, Salminen I, Lyssenko V, Virtanen SM, Groop L, Aro A, Saloranta C and the Botnia Research Group. The Pro12Ala polymorphism of the PPAR-γ2 gene affects associations of fish intake and marine *n*–3 fatty acids with glucose metabolism. Submitted.

III Ylönen K, Alfthan G, Groop L, Saloranta C, Aro A, Virtanen SM, and the Botnia Research Group. Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. Am J Clin Nutr 2003;77:1434–1441.[1]

IV Ylönen K, Saloranta C, Kronberg-Kippilä C, Groop L, Aro A, Virtanen SM and the Botnia Research Group. Associations of dietary fiber with glucose metabolism in nondiabetic relatives of subjects with type 2 diabetes. The Botnia Dietary Study. Diabetes Care 2003;26:1979–1985.[2]

In addition, some unpublished results are presented.

Abbreviations

BMI	body mass index (body weight [kg] divided by the square of height [m^2])
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GHb	glycosylated haemoglobin (HbA_{1c})
GLUT4	glucose transporter 4
HDL	high-density lipoprotein
HGP	hepatic glucose production
HOMA-IR	homeostasis model assessment - insulin resistance index
IFG	impaired fasting glucose
IGI	insulinogenic index
IGT	impaired glucose tolerance
IVGTT	intravenous glucose tolerance test
LDL	low-density lipoprotein
MET	metabolic equivalent
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
PPAR	peroxisome proliferator-activated receptor
PPARG	gene encoding for PPAR-γ
Pro12Ala	substitution of an alanine for a proline at amino acid 12 in *PPARG*
PUFA	polyunsaturated fatty acid
SD	standard deviation
SE	standard error
SFA	saturated fatty acid
T2D	type 2 diabetes
TG	triglyceride
TZD	thiazolidinedione
WHR	waist-to-hip ratio

1 Introduction

Type 2 diabetes (T2D) is a heterogeneous disorder of carbohydrate, lipid and protein metabolism. This chronic progressive disease results from complex interactions between genetics and early life and adult environmental influences. It is characterized by resistance to insulin-mediated glucose disposal in muscle and adipose tissue, failure of β-cells to compensate for this resistance and overproduction of glucose in the liver.

Both prevalence and incidence rates of T2D have been increasing worldwide over the past decades. As the genetic constitution of man has not changed rapidly enough to explain the diabetes epidemic, lifestyle-related factors seem to have a major impact. The imbalance between energy intake and energy expenditure, leading to obesity, is an important determinant of T2D. In addition, changes in the composition of the diet and interactions between genes and diet may play a role. Modern diets are low in *n*–3 fatty acids, antioxidative nutrients (*e.g.*, carotenoids and vitamin E) and fibre compared with ancient diets (Arab 2003, Simopoulos 2003, Cordain *et al.* 2005). The nutritional patterns in contemporary affluent nations may thus be poorly compatible with the older genome, thus contributing to the development of lifestyle-related diseases (Simopoulos 2003, Cordain *et al.* 2005).

Although T2D may be largely preventable through lifestyle intervention (Pan *et al.* 1997b, Tuomilehto *et al.* 2001, Knowler *et al.* 2002), the prevailing knowledge of the roles of many dietary components as risk or protective factors in the aetiology of T2D is contradictory. One underlying factor causing discrepancy in results may be genetic heterogeneity among study populations. Evidence on nutrient-gene interactions in relation to glucose metabolism is scarce. Increased understanding of dietary determinants of glucose metabolism as well as recognition of underlying interactions between genetic and dietary influences is crucial for the development of effective dietary prevention strategies to slow and reverse the disturbing T2D trends.

Peroxisome proliferator-activated receptors (PPARs) have recently emerged as one group of central regulators of nutrient-gene interactions. They belong to the nuclear hormone receptor superfamily and regulate genes controlling lipid and glucose metabolism as well as adipogenesis. The fact that structurally diverse fatty acids can act as ligands of nuclear receptors provides a direct link between the availability of fatty acids and gene expression. One of the PPAR subtypes, PPAR-γ, regulates the expression of an array of genes involved in adipocyte differentiation, lipid stor-

age and insulin sensitization. Currently, one of the most consistent genetic predictors of T2D is a common variant in the PPAR-γ2 isoform, which results in the substitution of an alanine for a proline at amino acid 12, producing Pro12Ala (Yen *et al.* 1997, Lohmueller *et al.* 2003). Studies on interactions between the Pro12Ala polymorphism and dietary fatty acids in glucose metabolism are thus far limited, but suggest an important role for this polymorphism in modulating the metabolic response to fatty acids (Luan *et al.* 2001, Robitaille *et al.* 2003, Pisabarro *et al.* 2004).

In this study, associations of dietary and plasma cholesterol ester fatty acids as well as those of dietary and plasma tocopherols and carotenoids and dietary fibre with glucose metabolism were examined in a high-risk population for T2D, *i.e.*, in non-diabetic relatives of affected patients. Interactions between fatty acids and the Pro12Ala polymorphism of the PPAR-γ2 gene in glucose metabolism were also investigated.

2 Review of the Literature

Diabetes mellitus is a phenotypically heterogeneous metabolic disease of complex aetiology, characterized by chronic hyperglycaemia (Alberti and Zimmet 1998). In addition to impaired carbohydrate metabolism, diabetes is associated with alterations in fat and protein metabolism.

The diagnosis of diabetes is based on blood glucose levels in the fasting state and at the 2-h time point during a standardized oral glucose tolerance test (OGTT) (Alberti and Zimmet 1998, World Health Organization 1999). According to the latest WHO criteria, diabetes is classified into four distinct types: types 1 and 2 (until recently, referred to as insulin-dependent and non-insulin-dependent diabetes, respectively), gestational diabetes mellitus and other specific types (Alberti and Zimmet 1998, World Health Organization 1999). Besides diabetes diagnosis, two pre-diabetic states, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), are defined. IGT represents hyperglycaemia with glucose values intermediate between normal and diabetic following a glucose load. IFG refers to elevated fasting glucose concentrations, which while not meeting the criteria for diabetes, are too high to be considered normal. Both IGT and IFG are associated with increased risk of future diabetes (Edelstein *et al*. 1997, Alberti and Zimmet 1998).

Of the two main diabetes types, type 1 develops due to autoimmune-mediated destruction of pancreatic β-cells, resulting in absolute insulin deficiency (Alberti and Zimmet 1998). Type 2 diabetes is characterized by insulin resistance and abnormal insulin secretion (Alberti and Zimmet 1998). Some of the specific subtypes of diabetes, such as maturity-onset diabetes of the young, are monogenic (Hattersley 1998, Hansen and Pedersen 2005), but mostly the pathogenesis involves multiple genes, environmental triggers and interactions between these (Groop and Tuomi 1997, McCarthy 2004). The following text deals only with type 2 diabetes.

2.1 Type 2 diabetes – an increasing health problem worldwide

Rising incidence and prevalence of type 2 diabetes

Globally, T2D accounts for about 90% of all diabetes cases (Stumvoll *et al*. 2005). In 1995, the prevalence of diabetes (all types) among the adult population aged ≥ 20 years was estimated to have been about 4% worldwide (King *et al*. 1998). In high-risk groups in Western countries, the prevalence

may reach 20%, and even higher figures have been found in some ethnic groups in North America and the Pacific Islands (Steyn *et al*. 2004). The prevalence and incidence rates of T2D have been increasing throughout the world during the past decades to epidemic proportions (Zimmet *et al*. 2001).

In 1995, the number of adults aged ≥ 20 years with diabetes was estimated to have been 135 million globally, and the figure was predicted to more than double from 1995 to 2025 to reach 300 million (King *et al*. 1998). According to another recent report, the estimates of subjects with diabetes were 171 million in 2000 and 366 million in 2030 (Wild *et al*. 2004). These figures are, however, based solely upon aging of the populations, and the increasing prevalence of obesity is likely to increase the estimates.

The number of subjects with T2D was over 160 000 in Finland in 2004 based on the Social Insurance Institution's nationwide register on reimbursement for diabetes medication (Klaukka 2005). The proportional increase from the year 2003 was 5.7%. According to King *et al*. (1998), the number of adults with diabetes, including both known and undiagnosed cases, in Finland in 1995 was 300 000, and the estimation for 2025 was 454 000. However, based on a recent Finnish cross-sectional population-based survey among subjects aged 45-74 years, the total prevalence of T2D, including both previously diagnosed cases and those detected at screening, was 16% in men and 11% in women (Peltonen *et al*. 2006). The prevalence of abnormal glucose tolerance, also including IFG and IGT, was 42% in men and 33% in women.

Until recent decades, T2D was a disease of the middle-aged and elderly. An emerging public health concern is the appearance of IGT and T2D among overweight children and adolescents, especially in high-risk populations (Rosenbloom *et al*. 1999, Fagot-Campagna *et al*. 2001). For example, in a multi-ethnic cohort of obese American children aged 4-10 years and adolescents aged 11-18 years with a body mass index (BMI) over 30 kg/m^2, IGT was diagnosed in 25% of children and 21% of adolescents (Sinha *et al*. 2002). Of the obese adolescents, 4% had silent T2D. Available data on the prevalence of T2D in European Caucasian children and adolescents suggest that the problem may be milder than in North America or in other races, but nevertheless more serious than thus far thought (Erhardt and Molnár 2004, Wiegand *et al*. 2004). Of Hungarian obese adolescents, for instance, ~ 18% had IGT and < 2% had T2D (Erhardt and Molnár 2004). Preliminary data show that IGT and T2D have been diagnosed in Finnish children as well (Saha *et al*. 2003).

The burden of type 2 diabetes

Subjects with T2D often exhibit an array of associated traits such as hypertension, dyslipidaemia and hypercoagulability. These contribute to the vascular complications of the disease, leading to premature morbidity

and mortality (Isomaa 2003, Parillo and Riccardi 2004, Steyn *et al*. 2004). Subjects with T2D have a 2- to 4-fold age-adjusted cardiovascular mortality compared with non-diabetic subjects (Haffner *et al*. 1998, Parillo and Riccardi 2004). Micro- and macrovascular complications are often present in individuals with newly diagnosed T2D, indicating that the onset of the disease may occur years – up to a decade – before the clinical diagnosis (Harris *et al*. 1992).

Besides human suffering, the economic burden of T2D and its complications is considerable. Health care systems worldwide will sustain enormous costs for the treatment of diabetes and its complications in the near future if the ever-increasing number of individuals diagnosed with the disease is not halted (Zimmet *et al*. 2001). Thus, strategies to slow or even reverse the escalating epidemic of T2D are clearly needed. Although this disease may be largely preventable (Pan *et al*. 1997b, Tuomilehto *et al*. 2001, Knowler *et al*. 2002), in order to develop the most successful preventive measures, it is essential to gain a more comprehensive understanding of the pathogenesis and underlying risk and protective factors. Whether dietary modifications required differ, for example, according to the genetic susceptibility of subjects remains obscure.

2.2 Metabolic abnormalities of type 2 diabetes

In healthy subjects, blood glucose levels are maintained within a narrow range by the coordinated interplay between glucose absorption and uptake, metabolism by peripheral tissues and hepatic glucose production (HGP) (Saltiel and Kahn 2001). Insulin, which is the main hormone for the regulation of blood glucose, is secreted from pancreatic β-cells in response to elevations in blood glucose concentrations, and exerts its effects by binding to and activating a specific plasma membrane receptor in peripheral target tissues. Insulin promotes glucose uptake in muscle and fat cells by stimulating the translocation of insulin-responsive glucose transporter 4 (GLUT4) from intracellular sites to the cell surface. Subsequently, insulin promotes glycolysis as well as storage of glucose and other substrates by stimulating glycogen, lipid and protein synthesis. In adipocytes, insulin also inhibits lipolysis. In hepatocytes, insulin stimulates lipid and glycogen synthesis and inhibits the production and release of glucose by suppressing glycogenolysis and gluconeogenesis.

The gradual deterioration of glucose homeostasis leading to T2D is characterized by an array of metabolic alterations, the main ones being peripheral insulin resistance, progressive deterioration in β-cell function and overproduction of glucose in the liver (Saad *et al*. 1991, Lillioja *et al*. 1993, Alberti and Zimmet 1998, Kahn 2003, Lyssenko *et al*. 2005).

2.2.1 Insulin resistance

Insulin resistance is defined as a metabolic state in which physiological concentrations of insulin produce a subnormal biological response that occurs at the level of all insulin-dependent tissues, including, but not limited to, muscle, adipose tissue and the liver (Caro 1991). As skeletal muscle accounts for ~ 80% of insulin-stimulated glucose utilization (Evans *et al*. 2004), it has been regarded as the major site of insulin resistance in T2D, manifested mainly as reduced non-oxidative glucose disposal (Eriksson *et al*. 1989, Petersen *et al*. 2004). In adipose tissue, insulin resistance leads to an excessive rate of lipolysis and ensuing elevated circulating non-esterified fatty acid (NEFA) concentrations (McGarry 2002, Kashyap *et al*. 2003). In the liver, glucose output is not suppressed as a result of insulin resistance.

Insulin sensitivity is influenced by a number of factors, such as age, genetics and the environment, as well as by the interaction between these (Groop and Tuomi 1997, Kahn 2003). At least partly lifestyle-determined factors known to promote insulin resistance include overall obesity, intra-abdominal fat accumulation, smoking, physical inactivity and certain dietary factors (Facchini *et al*. 1992, Rönnemaa *et al*. 1996, Bessesen 2001, Kahn 2003, Parillo and Riccardi 2004).

The mechanisms for the reduction in insulin sensitivity are not clearly understood, but are likely to include impaired access of insulin to skeletal muscle due to lowering of blood flow or endothelial insulin transport, defects in post-receptor insulin signalling, glucose transport or enzymes regulating intracellular glucose metabolism and mitochondrial dysfunction (Bergman and Ader 2000, Shulman 2000, Clark *et al*. 2003, Del Prato 2003, Petersen *et al*. 2004, Hansen and Pedersen 2005, Stumvoll *et al*. 2005). Insulin resistance associated with obesity may be caused by an excess production by hypertrophic adipocytes of NEFAs, inflammatory cytokines and adipokines (Kahn 2003, Stumvoll *et al*. 2005).

Insulin resistance is often accompanied by a cluster of metabolic abnormalities, including, in addition to glucose intolerance, central obesity, dyslipidaemia, hypertension, hypercoagulability and microalbuminuria (Reaven 1988, Alberti and Zimmet 1998). This clustering of risk factors is referred to as the 'metabolic syndrome', 'insulin resistance syndrome' or 'syndrome X' (Reaven 1988, Isomaa 2003).

2.2.2 Impaired β-cell function

Another metabolic abnormality typical of disordered glucose metabolism is the deterioration of β-cell function. This is manifested as diminution in the sensitivity of β-cells to glycaemic stimuli and decreased insulin secretion (Alberti and Zimmet 1998, Del Prato 2003, Ferrannini *et al*. 2003). As a result, insulin responses during OGTT, intravenous glucose tolerance test (IVGTT) and hyperglycaemic clamp are delayed and impaired (Eriksson

et al. 1989, Pimenta *et al*. 1995, Vaag *et al*. 1995, Alberti and Zimmet 1998, Kahn 2003).

Proposed mechanisms for diminution of insulin response comprise genetic defects in β-cell mass or function, damage of the secretory process due to prolonged exposure to elevated levels of glucose (glucotoxicity) or NEFA (lipotoxicity), or both, and amyloid deposition (Evans *et al*. 2002, El-Assaad *et al*. 2003, Steyn *et al*. 2004, Stumvoll *et al*. 2005, Poitout *et al*. 2006).

Because of the complex and long-lasting disease process through several stages, the metabolic pathways and the sequence of underlying defects which lead to T2D have remained unclear (Harris *et al*. 1992, Alberti and Zimmet 1998, McGarry 2002). Nevertheless, both insulin resistance and defects in insulin secretion are detectable in pre-diabetic states and in subjects with a genetic predisposition to the disease, and they also predict the development of clinically overt hyperglycaemia (Eriksson *et al*. 1989, Lillioja *et al*. 1993, Vaag *et al*. 1995, Perseghin *et al*. 1997, Alberti and Zimmet 1998, Kahn 2003, Lyssenko *et al*. 2005). The prevailing notion is that insulin resistance is compensated by augmented β-cell function, but if insulin resistance is not reversed, a chronically increased insulin demand leads to β-cell exhaustion in susceptible individuals, which can result in glucose intolerance (Saad *et al*. 1991, Kahn 2003, Lyssenko *et al*. 2005). However, dysfunction of insulin secretion without impairment of insulin sensitivity has been observed in first-degree relatives of subjects with T2D (Pimenta *et al*. 1995). The relative contributions of insulin resistance and insulin secretion defects in the pathophysiology of the disease may furthermore differ according to the ethnic background (Lillioja *et al*. 1993, Pimenta *et al*. 1995, Alberti and Zimmet 1998, Kahn 2003).

2.2.3 Hepatic glucose overproduction

The third main metabolic abnormality that characterizes T2D is the overproduction of glucose in the liver as a result of enhanced gluconeogenesis (Magnusson *et al*. 1992). This defect has, however, been regarded as a later secondary defect, as HGP and its suppression by insulin were normal in genetically predisposed twins (Vaag *et al*. 1995) and first-degree relatives of patients with T2D (Eriksson *et al*. 1989), and as the rate of HGP did not predict future T2D (Lillioja *et al*. 1993). In one study, by contrast, HGP was increased in non-diabetic relatives of patients with T2D (Osei 1990).

2.2.4 Disordered fat mobilization and storage

Besides hyperglycaemia, T2D is characterized by abnormal fat storage and mobilization, manifested as elevated fasting and post-prandial circulating NEFA and triglyceride (TG) levels as well as excessive deposition of fat in non-adipose tissues (Cortright *et al*. 1997, McGarry 2002). The failure of insulin to suppress lipolysis is observed in subjects genetically predisposed to T2D (Lewis *et al*. 2002), and elevated NEFAs are predictive of deteriora-

tion in glucose tolerance (Charles *et al.* 1997). Increased non-adipose tissue fat accumulation characterizes non-obese insulin-resistant offspring of parents with diabetes (Jacob *et al.* 1999, Perseghin *et al.* 1999).

The mechanisms through which excess NEFA concentrations may lead to insulin resistance and glucose intolerance are multiple. In 1963, Randle *et al.* proposed the operation of the glucose-fatty acid cycle in muscle cells according to which elevated NEFAs induce insulin resistance by competing with glucose for oxidation. More recent data have suggested that increased concentrations of NEFA may induce insulin resistance via inhibition of endothelial insulin transport, defects in insulin signalling and reduced skeletal muscle glucose uptake and glycogen synthesis (Dresner *et al.* 1999, Bergman and Ader 2000, Shulman 2000, Saltiel and Kahn 2001).

Acutely, NEFAs have been shown to enhance, but after a prolonged exposure to suppress insulin secretion (Grill and Qvigkstad 2000, Kashyap *et al.* 2003, Poitout *et al.* 2006). NEFAs may also induce β-cell apoptosis (Eitel *et al.* 2002). Increased exposure of the liver to NEFAs increases HGP, leads to excessive storage of TGs in liver, increases output of TG-rich very-low-density lipoproteins and interferes with hepatic insulin clearance, resulting in glucose intolerance, hypertriglyceridaemia and hyperinsulinaemia (Bergman and Ader 2000, Wajchenberg 2000). Finally, high NEFA may increase oxidative stress and activate, for example, the nuclear factor-κB signalling pathway, which has been speculated to be involved in the development of insulin resistance and impaired insulin secretion (Evans *et al.* 2002).

In the pathogenesis of T2D, increasing evidence supports a pivotal role for abnormal accumulation of TGs, fatty acids and their metabolites in non-adipose tissues, especially muscle and liver, and also in pancreatic β-cells; some data suggest that this might even be the main underlying disorder explaining both insulin resistance and impaired insulin secretion (Storlien *et al.* 1991, Cortright *et al.* 1997, Pan *et al.* 1997a, Grill and Qvigstad 2000, Shulman 2000, Wajchenberg 2000, Evans *et al.* 2002, 2004, Kelley *et al.* 2002, Lewis *et al.* 2002, Ravussin and Smith 2002, Petersen *et al.* 2004). This abnormality could be a consequence of increased delivery of fatty acids, impaired fatty acid oxidation or an imbalance between uptake and oxidation (Cortright *et al.* 1997, Kelley *et al.* 2002, Lewis *et al.* 2002, McGarry 2002).

Adipose tissue regulation of glucose homeostasis

Previously, the main role of white adipose tissue has been thought to be to store energy during times of plenty and to release energy during times of need. Recently, adipose tissue has been recognized to communicate continuously with other tissues (Evans *et al.* 2004). Under hormonal control, adipose tissue functions when it responds to insulin by increasing the differentiation of pre-adipocytes to adipocytes, by increasing glucose uptake and TG synthesis and by inhibiting lipolysis (Evans *et al.* 2004). As an endocrine organ, adipose tissue produces and secretes several hormones and

other protein factors called adipokines (Ravussin and Smith 2002, Trayhurn and Wood 2004). These include, for example, leptin, tumour necrosis factor-α, plasminogen activator inhibitor-I, adiponectin, resistin and interleukin-6 (Havel 2002, Ravussin and Smith 2002, Trayhurn and Wood 2004, Wolf 2004). These factors regulate the function of several other organs and affect the metabolism of the body overall (Morrison and Farmer 2001, Trayhurn and Wood 2004). Leptin reduces food intake and increases energy expenditure and insulin sensitivity (Havel 2002). Tumour necrosis factor-α affects insulin action, *e.g.*, via the inhibition of lipogenesis and stimulation of lipolysis, as well as directly by impairing insulin signalling (Trayhurn and Wood 2004, Wolf 2004). Plasminogen activator inhibitor-I is involved in the maintenance of vascular homeostasis (Trayhurn and Wood 2004). Adiponectin promotes fatty acid oxidation in skeletal muscle, increases insulin sensitivity and decreases HGP (Havel 2002, Holst and Grimaldi 2002). Resistin inhibits the differentiation of preadipocytes to adipocytes, increases adipose tissue and liver insulin resistance, reduces insulin-mediated glucose uptake and increases HGP and glucose levels (Wolf 2004). Markers of subclinical inflammation, such as interleukin-6, are elevated in insulin-resistant states and have predicted the development of T2D (Pradhan *et al.* 2001, Hu and Stampfer 2003). Thus, besides the deleterious effects of increased delivery of NEFAs, excessive adipose tissue in obesity may adversely affect glucose metabolism through dysregulated secretion of adipokines.

2.3 Aetiology of type 2 diabetes

According to the contemporary conceptual framework, T2D is a multifactorial disease resulting from a complex interaction between genetics, early life and adult environmental factors, leading to the presence of defects of glucose homeostasis (Figure 1).

The two major hypotheses put forward concerning the aetiology of T2D are the 'thrifty genotype' and the 'thrifty phenotype'. The 'thrifty genotype' hypothesis postulates that humans were programmed during the hunter-gatherer era for a thrifty response to energy supply (Neel 1962). Adaptive mechanisms that promoted effective energy storage and fat deposition during times of plenty and efficient use of energy during episodes of food shortage were selected to enhance the likelihood of survival (Neel 1962, Lewis *et al.* 2002). Under contemporary affluent conditions where energy-dense foods are readily available and do not require significant energy expenditure, these same responses have lost their usefulness and may result in obesity and T2D.

The 'thrifty phenotype' hypothesis, which was tendered by Hales and Barker (1992), was based on and later further supported by epidemiological observations linking low birth weight with the risk of adult disease,

including T2D (*e.g.*, Lithell *et al.* 1996, Ravelli *et al.* 1998; summarized by Phillips 1998 and Steyn *et al.* 2004). The hypothesis proposes that malnutrition during critical periods of intrauterine development and early life leads to permanent changes in the structure or function, or both, of the developing systems, a process called programming, which could predispose to disease in adult life (Hales and Barker 1992, Ozanne and Hales 1999). Regarding T2D, foetal malnutrition would, *e.g.*, lead to decreased β-cell mass and reduced insulin secretion (Hales and Barker 1992). Although the metabolic adaptations would be beneficial to survival in poor nutritional conditions, long-term modifications to organ structure, hormone responsiveness or gene expression might be detrimental and predispose to metabolic disorders when nutrition is abundant in later life (Leon 1998, Phillips 1998, Ozanne and Hales 1999, Simmons 2005).

The 'thrifty genotype' and 'thrifty phenotype' hypotheses are not necessarily exclusive and may complement each other (Frayling and Hattersley 2001, Jaquet *et al.* 2002). A study in twins provided evidence of a role for both the intrauterine environment and genetic factors in the aetiology of insulin resistance (Poulsen *et al.* 2003). Furthermore, genetically determined impairment in insulin action or secretion could result in low foetal growth and insulin resistance later on (Hattersley and Tooke 1999, Hansen and Pedersen 2005).

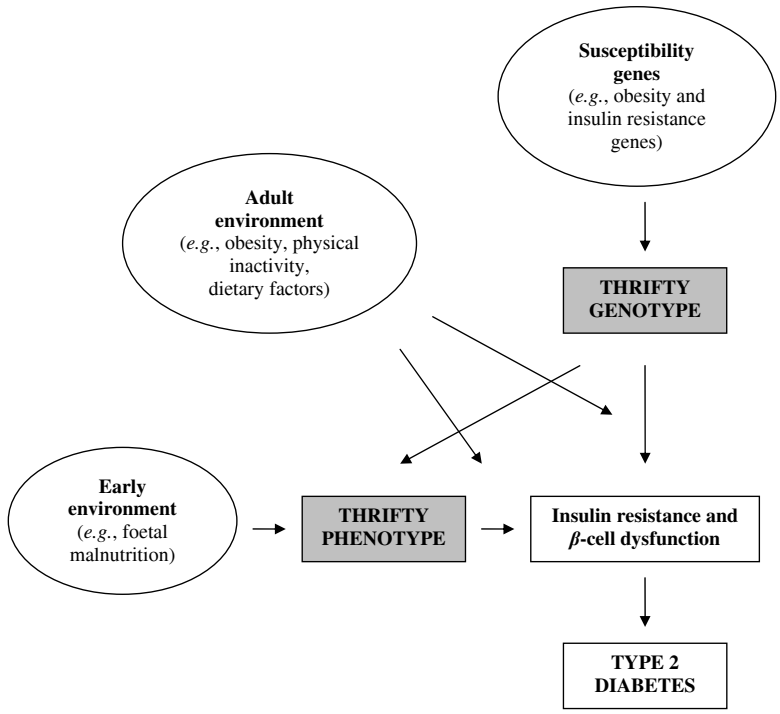

Figure 1. Aetiology of T2D (depicted according to Mandrup-Poulsen 1998, Frayling and Hattersley 2001, Steyn *et al.* 2004, Lee *et al.* 2005).

Aetiological determinants and risk factors of type 2 diabetes

Aetiological determinants and risk factors that contribute to the development of T2D include the following (Zimmet *et al*. 2001):

Genetic factors
- Genetic markers, family history, 'thrifty gene(s)'

Demographic characteristics
- Sex, age, ethnicity

Behavioural and lifestyle-related risk factors
- Obesity (including distribution of obesity and duration)
- Physical inactivity
- Diet
- Stress
- Westernization, urbanization, modernization

Metabolic determinants and intermediate risk categories of T2D
- IGT
- Insulin resistance
- Pregnancy-related determinants (parity, gestational diabetes, diabetes in offspring of women with diabetes during pregnancy, intrauterine mal- or overnutrition)

T2D aggregates in families (Yki-Järvinen 1994, Lyssenko *et al*. 2005). This is likely to be more the result of genetics than social origin, as monozygotic twins show higher concordance rates for the disease than dizygotic twins (Newman *et al*. 1987, Kaprio *et al*. 1992). In addition, 45% of first-degree relatives of patients with T2D are insulin-resistant compared with only 20% of individuals without a family history of diabetes matched for age and BMI (Groop *et al*. 1996). First-degree relatives also have an increased risk of developing the disease compared with the general population (Perseghin *et al*. 1997, Hansen and Pedersen 2005, Stumvoll *et al*. 2005). Migration studies add further to the evidence that genetic variation contributes to individual susceptibility to T2D (McCarthy 2004). The role of heredity appears, however, to differ between populations and to be dependent on the environment (O'Rahilly *et al*. 2005).

Regarding demographic determinants, the risk of developing T2D is known to increase with age (Alberti and Zimmet 1998, Steyn *et al*. 2004), and persons from certain ethnic backgrounds, such as African-Americans, Hispanics and Native Americans, are at greater risk than Caucasians (Haffner 1998, Steyn *et al*. 2004). Some findings suggest that women have a greater risk (Rosenbloom *et al*. 1999), although the sex difference may be explained by differences in, for instance, obesity and physical activity (Steyn *et al*. 2004).

The prevailing epidemic of T2D has arisen only over the past decades, and thus, cannot be due to changes in the human genome. Instead it reflects

marked changes in environmental factors, the importance of which in the pathogenesis of T2D is supported by not all monozygotic twins being concordant for the disease (Newman *et al*. 1987, Medici *et al*. 1999). Observations in twins have also shown that non-genetic components contribute to all three major metabolic abnormalities of T2D, *i.e*., insulin resistance, impaired insulin secretion and elevated HGP (Vaag *et al*. 1995).

2.3.1 Genetic factors

The factors and the extent to which they are inherited in T2D are largely obscure. Nevertheless, the disease is known to be complex, and a number of genes are likely to be involved, each with modest effects (Kadowaki *et al*. 2003, McCarthy 2004). These may include, but are not necessarily limited to, genes encoding for proteins that are involved in β-cell, myocyte, adipocyte and/or hepatocyte development and function, insulin signalling, intermediary metabolism or hypothalamic regulation (Saltiel and Kahn 2001, McCarthy 2004). Both insulin sensitivity and insulin secretion are partly under genetic control (Vauhkonen *et al*. 1998, Lehtovirta *et al*. 2000, Hansen and Pedersen 2005).

Recent advancements in genomic research and statistical methods have enabled attempts to unravel genetic variants that influence T2D-related traits and predispose to the disease. The candidate gene approach examines *a priori* selected genes with either an identified or a suspected role in the disease process, while the genome-wide scan or linkage analysis refers to the systematic screening of the entire genome to identify novel genes that influence the phenotype of interest (Stumvoll *et al*. 2005).

Over the past years, several genes regulating fat storage and distribution, NEFA metabolism, fuel oxidation and skeletal muscle glucose metabolism have been investigated for their potential role in T2D, but no genetic locus accounting for the disease has been found (Isomaa 2003). Similarly, several studies have suggested an association between random genetic markers and T2D; however, for many of them, there is a lack of replication in subsequent analyses (Rosenbloom *et al*. 1999, O'Rahilly *et al*. 2005, Willer *et al*. 2007). Currently, the most significant susceptibility genes of T2D seem to be 1) the *ABCC8* and *KCNJ11* genes encoding sulfonylurea receptor 1 (SUR1) and inwardly rectifying K^+ channel (Kir6.2) subunits of the β-cell ATP-sensitive K^+ channel, which plays a central role in glucose-stimulated insulin secretion (Inoue *et al*. 1996, Hani *et al*. 1998; summarized by van Dam *et al*. 2005); 2) the *PPARG* gene encoding a transcription factor that is involved in adipocyte differentiation and regulation of glucose and lipid homeostasis (Altshuler *et al*. 2000); and 3) the *CAPN10* gene encoding cysteine protease calpain-10, which affects a variety of cellular functions and may play a role in insulin secretion, insulin action and HGP (Horikawa *et al*. 2000). Of these, the strongest evidence for replication has been documented for the Pro12Ala polymorphism in the *PPARG*, and for the Glu-

23Lys polymorphism in *KCNJ11* (Gloyn *et al*. 2003, Lohmueller *et al*. 2003, van Dam *et al*. 2005, Willer *et al*. 2007). Very recently, however, a strong association between variants in the *TCF7L2* gene, encoding a transcription factor, and T2D was reported by Grant *et al*. (2006); with replication of the finding in several populations (Cauchi *et al*. 2006a, b, Damcott *et al*. 2006, Groves *et al*. 2006, Munoz *et al*. 2006, Saxena *et al*. 2006, Scott *et al*. 2006, Zhang *et al*. 2006). The mechanisms by which the variants in *TCF7L2* affect glucose metabolism are at the moment unclear, but suggested mechanisms include effects on insulin secretion via the regulation of glucagon-like peptide-1, and the regulation of genes involved in adipogenesis and/or adipocyte function (Damcott *et al*. 2006, Grant *et al*. 2006).

Regarding the unravelling of possible nutrient-gene interactions in glucose metabolism, of the prevailing susceptibility genes of T2D, *PPARG* is of special interest, as fatty acids have been shown to be the natural ligands of PPARs (Forman *et al*. 1997, Kliewer *et al*. 1997, Krey *et al*. 1997, Xu *et al*. 1999), and as some results suggest an interaction between fatty acids and the Pro12Ala polymorphism of *PPARG* with respect to glucose metabolism (Luan *et al*. 2001, Robitaille *et al*. 2003, Pisabarro *et al*. 2004).

2.3.1.1 Peroxisome proliferator-activated receptors as regulators of glucose metabolism

PPARs are nuclear receptors that function as ligand-activated transcription factors. They control the expression of numerous genes encoding for proteins involved in multiple pathways of energy, fatty acid and glucose metabolism, as well as in inflammation and cell differentiation (Berger and Moller 2002, Wolf 2003, Fredenrich and Grimaldi 2004, Berger *et al*. 2005).

The three PPAR subtypes, PPAR-α, PPAR-δ (also known as PPAR-β) and PPAR-γ, are encoded by three different genes, and their tissue distribution shows a distinct pattern (Jump and Clarke 1999, Evans *et al*. 2004). PPAR-α is predominantly present in liver, while PPAR-δ is expressed in numerous tissues, albeit most abundantly in muscle and white adipose tissue (Wolf 2003, Evans *et al*. 2004). At least four isoforms of PPAR-γ (PPAR-γ1, -γ2, -γ3 and -γ4) have been identified (Sundvold and Lien 2001). Of these, PPAR-γ1 is expressed in many tissues (Ordovas and Corella 2004), while PPAR-γ2 is mainly expressed in adipose tissue, although detectable also at lower levels in, for instance, the pancreas (Lupi *et al*. 2004).

PPARs regulate the expression of target genes by dimerizing with another nuclear receptor, retinoid X receptor (activated by 9-*cis*-retinoic acid), and by binding to a specific DNA sequence termed the peroxisome proliferator response element, which is located in the promoter region of target genes (Morrison and Farmer 2001, Shulman and Mangelsdorf 2005). PPARs exert their effects on gene expression through ligand-dependent binding of coactivator and corepressor proteins (Stanley *et al*. 2003, Shul-

man and Mangelsdorf 2005). The binding of ligands to PPARs triggers a conformational change in the receptor, which results in the release of corepressors and an interaction with coactivators, leading to activation of the cellular transcriptional machinery (Berger *et al.* 1996, Stanley *et al.* 2003, Shulman and Mangelsdorf 2005).

PPARs have a large hydrophobic ligand-binding cavity, allowing them to bind a variety of ligands (Stanley *et al.* 2003). The receptors can be activated by saturated and unsaturated fatty acids, fatty acid derivatives, such as eicosanoids, and synthetic compounds (Forman *et al.* 1997, Kliewer *et al.* 1997, Krey *et al.* 1997, Xu *et al.* 1999, Evans *et al.* 2004, Shulman and Mangelsdorf 2005). PPAR-α, for example, is activated by lipid-lowering fibrate drugs (Kersten *et al.* 2000), and PPAR-γ by the antidiabetic thiazolidinediones (TZDs) (Berger *et al.* 2005). Importantly, plasma and tissue fatty acid levels in the human body are sufficiently high for PPAR activation (Jump and Clarke 1999, Morrison and Farmer 2001). Therefore, the PPARs provide a molecular link between dietary fatty acids and gene expression (Morrison and Farmer 2001).

PPAR-α appears to promote fatty acid oxidation in liver, while PPAR-δ enhances fatty acid oxidation and energy uncoupling in skeletal muscle and adipose tissue (Wolf 2003). These effects lead to lowered serum TG and NEFA levels, decreased lipid accumulation in muscle and liver and increased insulin sensitivity (Tanaka *et al.* 2003, Wolf 2003, Fredenrich and Grimaldi 2004, Berger *et al.* 2005, Grimaldi 2005).

PPAR-γ activation has been shown to promote adipogenesis, improve insulin sensitivity and reduce hyperglycaemia (Auwerx 1999). The tissue distribution of the receptor suggests that the effects are exerted primarily through adipose tissue, where it promotes preadipocyte differentiation into mature, TG-containing fat cells and promotes lipid storage in mature adipocytes (Rocchi and Auwerx 1999, Wolf 2003). As a result, lipid uptake and fatty acid storage in fat depots is enhanced, while lipolysis and NEFAs and TGs in the circulation, liver and muscle are diminished, thereby improving insulin sensitivity (McGarry 2002, Berger *et al.* 2005). The activation of PPAR-γ leads also to enhancement of insulin-stimulated adipocyte glucose uptake via increased expression of GLUT4 and c-Cbl-associated protein, which is involved in the translocation of GLUT4 to the cell surface (Rangwala and Lazar 2004).

The activation of PPAR-γ modulates the endocrine activity of adipose tissue, resulting in changes in adipokine production that affect glucose metabolism in hepatic and peripheral tissues (Khan and Vanden Heuvel 2003, Berger *et al.* 2005). For example, resistin, tumour necrosis factor-α and interleukin-6, which promote insulin resistance, are downregulated in response to PPAR-γ activation, while the expression of adiponectin, which potentiates insulin sensitivity, is upregulated (Havel 2002, Rangwala and Lazar 2004, Trayhurn and Wood 2004, Wolf 2004, Berger *et al.* 2005).

Although PPAR-γ is expressed at extremely low levels in muscle, it seems to be important for the maintenance of whole-body insulin sensitivity, as muscle-specific PPAR-γ deletion was shown to cause insulin resistance in mice (Hevener *et al.* 2003, Norris *et al.* 2003). Furthermore, PPAR-γ, with predominance of the PPAR-γ2 isoform, is expressed in pancreatic islets, and its activation may play a role in preventing the untoward effects of increased NEFA levels on insulin secretion (Lupi *et al.* 2004).

To summarize, PPAR-γ has the potential to influence glucose metabolism through a variety of mechanisms, highlighting the importance of further studying its role in the pathogenesis of T2D.

Pro12Ala polymorphism of the PPAR-γ2 gene

Among the most consistent genetic predictors of T2D to date is the Pro12Ala polymorphism in the PPAR-γ2 gene (*PPARG*), which results in the substitution of an alanine for a proline due to a *C*CG-to-*G*CG missense mutation at codon 12 of exon 6 (Yen *et al.* 1997, Lohmueller *et al.* 2003, Hansen and Pedersen 2005). Although the first results concerning the Pro12Ala variant with respect to the risk of T2D were discrepant, results of larger studies and a meta-analysis have shown that the *Pro/Pro* allele is associated with an ~ 1.25-fold increased risk for T2D (Altshuler *et al.* 2000, Lohmueller *et al.* 2003). As the diabetes-related high-risk allele is present in > 75% of individuals, this variant accounts for ~ 25% of the population-attributable risk of T2D (Altshuler *et al.* 2000, McCarthy 2004, Hansen and Pedersen 2005).

In functional studies, the Pro12Ala substitution has reduced the binding affinity of the PPAR-γ2 protein to peroxisome proliferator response element in target genes and decreased its transcriptional activity (Deeb *et al.* 1998). Like heterozygous PPAR-γ deficiency in mice (Miles *et al.* 2000), the reduced transcriptional activity in the *Ala* allele is associated with enhanced insulin sensitivity in humans (Deeb *et al.* 1998, Buzzetti *et al.* 2004). This may partly be due to reduced adipose tissue accumulation, as the *Ala* variant has been associated with lower BMI, although opposite observations have also been reported (Deeb *et al.* 1998, Berg 1999). In addition, the *Ala* allele has been associated with reduced NEFA delivery (Stumvoll *et al.* 2001) and increased insulin clearance (Tschritter *et al.* 2003), which probably contribute to the decreased T2D risk associated with the allele. The physiological consequences of the Pro12Ala polymorphism seem to be complex (Ordovas and Corella 2004); they may, for example, be different in lean and obese subjects (Rocchi and Auwerx 1999). Furthermore, the *Ala* variant may predispose to long-term weight gain (Lindi *et al.* 2001), and be a risk factor for insulin deficiency in subjects with T2D (Mori *et al.* 2001), although T2D subjects with this variant may also be more responsive to rosiglitazone treatment (Kang *et al.* 2005). Interestingly, both activation and partial loss of PPAR-γ may increase insulin sensitivity (Yamauchi *et al.* 2001).

As T2D is a multifactorial disease arising from interactions between genetic polymorphisms and environmental factors, it is of the utmost importance to unravel how glucose metabolism is affected by the interaction of common variants, including the *PPARG* Pro12Ala polymorphism, with environmental factors (McCarthy 2004). Some results suggest an interaction between the Pro12Ala polymorphism and physical activity on glucose metabolism (Adamo *et al.* 2005, Weiss *et al.* 2005). The few studies conducted thus far on an interaction between the Pro12Ala polymorphism and dietary fatty acids with respect to glucose metabolism are discussed later (see Section 2.3.3.1.).

2.3.2 Obesity and physical inactivity

Obesity and a low level of physical activity typical for sedentary urban environments represent the most important modifiable risk factors for T2D (Parillo and Riccardi 2004, Steyn *et al.* 2004). Longitudinal studies have shown that weight gain is a powerful predictor of the disease (Helmrich *et al.* 1991, Colditz *et al.* 1995, Hu *et al.* 2001a). Both overall and abdominal adiposity independently increase the risk (Parillo and Riccardi 2004, Wang *et al.* 2005). And, conversely, weight loss and increased physical activity are effective ways to prevent or delay the development of T2D (Helmrich *et al.* 1991, Colditz *et al.* 1995, Pan *et al.* 1997b, Tuomilehto *et al.* 2001, Knowler *et al.* 2002, Parillo and Riccardi 2004, Laaksonen *et al.* 2005).

2.3.3 Dietary factors

For decades, diet has been assumed to play an important role in the defects of glucose metabolism leading to T2D, as major alterations have been taking place in diets concomitantly with increasing T2D rates (Himsworth 1935, Bennett *et al.* 1984, Simopoulos 2003). The nutritional requirements of man have been proposed to be shaped according to the foods available to pre-agricultural humans (lean meat, fish, green leafy vegetables, fruits, nuts, berries and honey), and therefore, the nutritional patterns in contemporary affluent nations are poorly compatible with the ancient genome (Simopoulos 2003, Cordain *et al.* 2005). Among nutritional characteristics that have altered considerably over time are changes in the intake of the amount and type of fat, antioxidative nutrients (*e.g.*, carotenoids and vitamin E) and dietary fibre (Arab 2003, Simopoulos 2003, Cordain *et al.* 2005). Modern societies are characterized by sedentary lifestyles, leading to an imbalance between energy intake and energy expenditure, and contemporary diets are low in *n*–3 fatty acids, natural antioxidants and fibre (Simopoulos 2003). Affluent diets might also be poorly compatible with the thrifty metabolism programmed by a thrifty intrauterine environment.

The following text will provide a brief summary of the current knowledge of the associations between glucose metabolism and dietary fatty acids, tocopherols, carotenoids and fibre. Regarding fatty acids and anti-

oxidative nutrients, the associations between their plasma/tissue levels as surrogates of dietary intake and glucose metabolism are also discussed. The emphasis is primarily on results of epidemiological studies and intervention trials, but findings from *in vivo* and *in vitro* experimental studies are also presented when discussing potential mechanisms by which these nutrients influence glucose metabolism.

2.3.3.1 Fatty acids

Dietary fatty acids are classified as saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) on the basis of the number of double bonds in their hydrocarbon chain. The metabolism of fatty acids in the body is catalysed by elongases, which add carbon atoms to the carbon chain, and by desaturases, which introduce double bonds (Nakamura and Nara 2004). Delta-9 desaturase (also called stearoyl-CoA desaturase, SCD) catalyses the conversion of SFA precursors to MUFAs, while Δ6- and Δ5-desaturases catalyse the synthesis of very-long-chain unsaturated fatty acids. The activity of desaturases is mainly regulated by dietary PUFAs (all three desaturases are downregulated by high PUFA intake) (Nakamura and Nara 2004), but also by several hormones (Guéraud and Paris 1997). Many of the effects of fatty acids are dependent on their chain length and the number and position of double bonds (Stubbs and Smith 1990).

PUFAs occur in two functionally distinct families, the *n*–6 PUFAs and the *n*–3 PUFAs (a fatty acid with the first double bond after the third carbon from the methyl end is termed *n*–3). As *n*–6 and *n*–3 fatty acids compete for the same enzymes (Holman 1986, Cook and Spence 1987), a high intake of one can inhibit elongation and desaturation of the other. Although the liver has been regarded as the main site for long-chain PUFA production, desaturases are also expressed in other tissues, including the brain, liver, lung, heart, skeletal muscle and pancreas (*β*-cells) (Cho *et al*. 1999a, b, Ramanadham *et al*. 2002). Furthermore, human Δ5-desaturase is highly expressed in the foetal liver (Cho *et al*. 1999b, Leonard *et al*. 2000).

Putative mechanisms by which amount and composition of fat affect glucose metabolism

Fatty acids have several functions in the human body by which they may affect glucose metabolism. Fatty acids provide an efficient energy supply, but excessive fat accumulation is associated with increased NEFA levels, which may contribute to fat accumulation in non-adipose tissues (Kragen *et al*. 1991), and thereby, to decreased glucose uptake and oxidation in muscle (Storlien *et al*. 1991, 1996a). Among the different fatty acids, SFAs and *n*–6 PUFAs have been linked to accumulation of muscle triglycerides, while PUFAs of the *n*–3 series seem to direct fatty acids towards fatty acid oxidation (Storlien *et al*. 1991, Cortright *et al*. 1997, Clarke 2000, Lombardo and Chicco 2006). *N*–3 fatty acids have been associated with lower

overall and central adiposity (Hill *et al*. 1993, Takahashi and Ide 2000, Jang *et al*. 2003, Lombardo and Chicco 2006). Excessive adiposity may induce insulin resistance also by altered delivery of fat-derived adipokines (Trayhurn and Wood 2004).

The proportion of fat in the diet has been directly related to insulin resistance and the deterioration of glucose tolerance, especially in experimental animals (Kragen *et al*. 1991, Storlien *et al*. 1996a), but also in man, albeit more inconsistently (Storlien *et al*. 1996a, Parillo and Riccardi 2004). There are some indications that in man only a long-term exposure to a high-fat diet induces insulin resistance (Storlien *et al*. 1996a). The untoward effects of high fat intake on glucose metabolism seem to be partly mediated by obesity (Parillo and Riccardi 2004), but other mechanisms, investigated mainly in animal studies, are also evident. A high-fat diet may increase insulin release and lead to hyperinsulinaemia, with an ensuing decrease in the number of insulin receptors (Clarke 2000 and references therein). A high intake of fat is also associated with decreased insulin binding to its receptors and impaired insulin signalling (reviewed by Storlien *et al*. 1996a, Clarke 2000). Studies in rats have furthermore shown that high-fat diets downregulate GLUT4 expression and activity, although the decreases can be attenuated with high-fat diets rich in *n*–3 PUFAs (Rosholt *et al*. 1994, Takahashi and Ide 2000). Finally, high-fat diets have been shown to be associated with reduced glycogen synthase in mouse skeletal muscle (Storlien *et al*. 1996a) and downregulation of the genes involved in oxidative capacity in human and mouse skeletal muscle (Sparks *et al*. 2005).

The fatty acid composition of plasma and organelle membranes plays an important role in membrane function (Storlien *et al*. 1996a, b, Wiseman 1996, Vessby *et al*. 2002). SFAs with single bonds introduce rigidity to cell membranes, thereby impairing various membrane functions such as insulin receptor binding and the translocation and activity of glucose transporters (Storlien *et al*. 1996a). Double bonds of unsaturated fatty acids contribute to the fluidity of membranes with beneficial effects on glucose metabolism (Stolien *et al*. 1996b), but are also more chemically reactive than single bonds, thus being able to produce a variety of oxidized products that contribute to oxidative stress and membrane damage (see Section 2.3.3.2.). Fatty acids and their metabolites are furthermore used as modulators of cell signalling (Sampath and Ntambi 2005), and oxidative derivatives of fatty acids called eicosanoids are involved in, for example, inflammation (Funk 2001) and vasodilatation of blood vessels (Arab 2003).

Fatty acids influence insulin secretion. The effects seem to be dependent on chain length and the degree of unsaturation, may be dependent on whether studied *in vitro* or *in vivo* and may differ acutely and in long term (Opara *et al*. 1994, Stein *et al*. 1997, McGarry and Dobbins 1999, Beysen *et al*. 2002). The insulinotropic effect of fatty acids *in vitro* appears to increase as a function of chain length (Stein *et al*. 1997), although the results are

not all congruent (Opara *et al.* 1994). Regarding the degree of saturation, findings of *in vitro* studies are contradictory, showing increased secretion both with the degree of saturation (Stein *et al.* 1997) and with an increasing number of double bonds (Opara *et al.* 1994). In rats, fish oil feeding normalized the impaired insulin secretion caused by a sucrose-rich diet (Pighin *et al.* 2003). *In vivo* in humans, the insulin response to glucose after an acute elevation of plasma NEFAs was highest for MUFAs and lowest for SFAs (Beysen *et al.* 2002). Over time, however, a high ratio of saturated to polyunsaturated NEFAs in the blood may induce hypersecretion of insulin (McGarry and Dobbins 1999). Finally, SFAs, possibly in concomitance with elevated glucose concentrations, have been shown to impair β-cell secretory function through reduction of proliferative capacity and induction of β-cell death; unsaturated fatty acids, on the other hand, are protective and may prevent the deleterious effects of SFAs (Eitel *et al.* 2002, El-Assaad *et al.* 2003, Maedler *et al.* 2003).

Dietary and plasma fatty acid composition is associated with low-grade inflammation, which has recently been observed to be related to insulin resistance and T2D (Pannacciulli *et al.* 2001, Pradhan *et al.* 2001, Hu and Stampfer 2003, Weigert *et al.* 2004). High levels of SFAs have been associated with increased levels and PUFAs from the *n*–3 series with decreased levels of C-reactive protein and interleukin-6 (Fernández-Real *et al.* 2003, Rallidis *et al.* 2003, Simopoulos 2003, Bemelmans *et al.* 2004, Weigert *et al.* 2004, Ajuwon and Spurlock 2005, Klein-Platat *et al.* 2005). Results concerning *n*–6 fatty acids have been less consistent; both anti- and proinflammatory associations have been reported (Fernández-Real *et al.* 2003, Simopoulos 2003, Weigert *et al.* 2004, Klein-Platat *et al.* 2005, Sampath and Ntambi 2005).

Finally, fatty acids have the potential to affect glucose metabolism by regulation of gene expression via, for instance, membrane composition, intracellular calcium levels and eicosanoid production (Jump and Clarke 1999, Khan and Vanden Heuvel 2003, Sampath and Ntambi 2005). Importantly, fatty acids and their metabolites have been observed to regulate gene expression also directly via nuclear receptors, such as liver X receptors, hepatocyte nuclear factor-4α and PPARs, all of which are involved in glucose metabolism (Jump and Clarke 1999, Khan and Vanden Heuvel 2003, Sampath and Ntambi 2005).

Prevailing notion about relationships between fatty acids and glucose metabolism

Associations of dietary fat amount and composition with glucose metabolism in man have been investigated during recent decades in numerous studies with different study designs. Findings from observational epidemiological studies and controlled intervention trials were summarized in a recent report of joint WHO/FAO expert consultation on diet, nutrition

and prevention of chronic diseases (World Health Organization 2003, see also Steyn *et al*. 2004). As regards dietary fat, the strength of evidence available thus far was seen as probable for SFAs to increase the risk of T2D, as possible for high total fat intake and *trans* fatty acids to increase the risk and as possible for *n*–3 PUFAs to reduce the risk. According to a review by Parillo and Riccardi (2004), based primarily on epidemiological studies, a high intake of unsaturated fatty acids may potentially be associated with a decreased risk of T2D, and those of saturated and *trans* fatty acids with an increased risk.

The fatty acid composition in serum lipids and skeletal muscle membranes typical of insulin-resistant and diabetic states has usually been high in palmitic and palmitoleic acid, but low in unsaturated fatty acids (Vessby *et al*. 2002, Vessby 2003). These associations have been interpreted to be mainly explained by the fatty acid composition of the diet, although other factors may also play a role.

Some of the discrepancy in the results concerning associations between dietary fat and glucose metabolism is probably due to such methodological issues as population differences, sample size, limitations of methods used and different control for potential confounding factors. However, T2D is a multifactorial disease in which the phenotype of a subject is defined by interaction of genetic polymorphisms with environmental factors. Therefore, the failure to take into account the genetic variation among study subjects may be one factor underlying the inconsistent results (Bessesen 2001). Limited data thus far have suggested an interaction between dietary fat composition and polymorphisms linked to elevated TG concentrations (SstI polymorphism in the apolipoprotein C-III gene) (Salas *et al*. 1998), insulin resistance (Ala54Thr polymorphism in the intestinal fatty acid binding protein *FABP2*) (Marín *et al*. 2005) and coronary heart disease (–219G/T polymorphism in the apolipoproteinE gene promoter) (Moreno *et al*. 2005) with respect to associations with glucose metabolism.

Fatty acids probably exert some of their regulatory functions on metabolism through PPARs, for which they are natural ligands (Kliewer *et al*. 1997, Auverx 1999, Xu *et al*. 1999). The limited data available thus far suggest that the *PPARG* Pro12Ala genotype may be an important modifier of human physiological responses to dietary fat. Nieters *et al*. (2002) found an interaction between dietary arachidonic acid and the Pro12Ala polymorphism on the risk for obesity; only carriers of the *Ala* allele were at greater risk with increasing arachidonic acid intake. In the Nurses' Health Study population, the Pro12Ala polymorphism modified associations of total and monounsaturated fat with BMI (Memisoglu *et al*. 2003); a direct trend was observed between the intake of total fat and BMI in *Pro/Pro* homozygotes, but not in *Ala* allele carriers, while the intake of monounsaturated fat was inversely associated with BMI in *Ala* allele carriers, but not in *Pro/Pro* homozygotes. Furthermore, in carriers of the *Ala* allele, the de-

cline in serum TGs in response to fish oil supplementation may be greater (Lindi *et al.* 2003).

Results about interactions between dietary fatty acids and the *PPARG* Pro12Ala polymorphism on glucose metabolism are limited. In the study of Luan and co-workers (2001), the ratio of dietary PUFAs to SFAs was inversely related to the fasting insulin concentration in *Ala* allele carriers, but not in *Pro/Pro* homozygotes. In a later report by these investigators, the inverse association between the ratio of dietary PUFAs to SFAs and the fasting insulin concentration was only found in physically active *Ala* allele carriers (Franks *et al.* 2004). Pisabarro *et al.* (2004) describe a high intake of *trans* fatty acids, as well as of SFAs, to be associated with glucose intolerance only in the *Ala* allele carriers. Robitaille *et al.* (2003), by contrast, report a direct correlation of saturated fat intake with fasting glucose in *Pro/Pro* homozygotes, but not in *Ala* allele carriers. Reasons for these discrepancies are at present obscure, and the topic clearly warrants further attention.

2.3.3.2 Tocopherols and carotenoids

Tocopherols

Vitamin E is a fat-soluble vitamin that occurs in nature in eight structurally related forms: α-, β-, γ- and δ-tocopherol, and α-, β-, γ- and δ-tocotrienol (Brigelius-Flohé *et al.* 2002). In the circulation, vitamin E is transported in plasma lipoproteins, and its plasma concentrations are controlled by the liver in association with the control of lipoprotein metabolism (Traber and Arai 1999).

A high intake of vitamin E has been suggested to be protective against many chronic diseases, including T2D (Brigelius-Flohé *et al.* 2002). As vitamin E is a lipophilic antioxidant, which protects membranes from lipid peroxidation, the putative beneficial effects have been mainly ascribed to the antioxidative properties (Caballero 1993). Oxidative stress refers to the imbalance between the generation of highly reactive nitrogen and oxygen species and their removal through the antioxidant defence systems. It leads to tissue damage (Wiseman 1996) and has been linked to the impairment of insulin action and β-cell function, as well as to the development of T2D (Paolisso and Giugliano 1996, Facchini *et al.* 2000, Evans *et al.* 2002, Urakawa *et al.* 2003, Ceriello and Motz 2004). The effects would be mediated, *e.g.*, through decreased membrane fluidity, resulting in altered activity of the insulin receptor and interference with glucose uptake, or through common stress-activated signalling pathways such as nuclear factor-κB (Caballero 1993, Wiseman 1996, Evans *et al.* 2002). The trigger of oxidative stress has been speculated to be elevated NEFA or glucose levels, or both (Evans *et al.* 2002, Lewis *et al.* 2002, Ceriello and Motz 2004, Poitout *et al.* 2006).

Besides antioxidant effects, tocopherols have been shown to regulate cell signalling and gene expression, to affect inflammation and to play a role in the preservation of endothelial function (Azzi *et al*. 2000, Jiang *et al*. 2000, Laight *et al*. 2000, Brigelius-Flohé *et al*. 2002, Jiang and Ames 2003, Wagner *et al*. 2004, Wu *et al*. 2005). Some of the beneficial effects of vitamin E on glucose metabolism may thus occur via the inhibition of protein kinase C, possibly through nuclear factor-κB inhibition (Traber and Packer 1995, Azzi *et al*. 2000, Brigelius-Flohé *et al*. 2002, Evans *et al*. 2002). γ-Tocopherol could influence glucose metabolism through anti-inflammatory effects (Jiang *et al*. 2000, Jiang and Ames 2003, Wagner *et al*. 2004). Vitamin E may furthermore play a role in the preservation of insulin action through the maintenance of endothelial function (Laight *et al*. 2000). Endothelial dysfunction has recently been linked to abnormal glucose homeostasis (Vehkavaara *et al*. 1999, Hu and Stampfer 2003).

Beneficial effects on glucose metabolism have been supported by some (Paolisso *et al*. 1993, 1994, 1995), but not all (Meydani *et al*. 1994, 1998, Miller *et al*. 1997) human studies that have investigated the effects of pharmacological vitamin E doses (*e.g*., 800 mg/d). Evidence from epidemiological studies is also inconsistent. In one study, vitamin E intake was directly related to insulin sensitivity in vitamin E supplement users (Facchini *et al*. 1996), while in another the intake of vitamin E from food and supplements was unrelated to insulin sensitivity after adjustment for energy intake and BMI (Sanchez-Lugo *et al*. 1997). The intake of vitamin E has been both inversely related (Boeing *et al*. 2000, Bates *et al*. 2004) and unrelated (Shoff *et al*. 1993) to glycated haemoglobin (GHb), and one cohort study found dietary intakes of α-, γ- and δ-tocopherol as well as that of β-tocotrienol to be inversely related to the risk of T2D (Montonen *et al*. 2004).

The concentrations of plasma vitamin E, α-tocopherol and δ-tocopherol have been inversely associated with insulin resistance and insulin levels in several studies (Öhrvall *et al*. 1993, Decsi *et al*. 1996, Facchini *et al*. 2000), as well as with glucose concentration in one study (Ford *et al*. 2005). In the latter study, γ-tocopherol concentration, by contrast, was directly related to glucose concentration and GHb. A similar direct association between plasma γ-tocopherol and GHb was observed by Bates *et al*. (2004). In one cohort study, low plasma vitamin E concentration was associated with increased risk of T2D (Salonen *et al*. 1995), but in another longitudinal study, the inverse association between serum α-tocopherol and the risk of T2D was abolished after adjustment for cardiovascular risk factors (Reunanen *et al*. 1998). Mayer-Davis *et al*. (2002) found in their follow-up study that in vitamin E supplement non-users the dietary intake of vitamin E was unrelated to T2D incidence, while a high plasma α-tocopherol level showed a protective association. In vitamin E supplement users, by contrast, no protective effect was observed for either reported intake of vitamin E or the plasma concentration of α-tocopherol.

Based on the data available until the year 2003, the conclusion of the joint WHO/FAO expert consultation on diet, nutrition and prevention of chronic diseases was that vitamin E could decrease the risk of T2D, but the evidence was insufficient (World Health Organization 2003). Thereafter, however, results of two intervention studies have been reported. According to Czernichov *et al.* (2006), neither baseline dietary or plasma vitamin E nor supplementation with antioxidant vitamins and minerals, including 30 mg of vitamin E for 7.5 years, had any effect on fasting plasma glucose concentration. In the Women's Health Study, 600 IU of vitamin E every other day for 10 years also had no impact on the risk of T2D (Liu *et al.* 2006).

Carotenoids

Carotenoids are a family of natural pigments with hundreds of members. Of the major dietary carotenoids present in vegetables and fruits, α- and β-carotene exhibit, while lycopene is devoid of provitamin A activity (Rock *et al.* 1996, Stahl and Sies 1996, 1997, Clinton 1998). Carotenoids are transported in the circulation by lipoproteins, and they concentrate in certain tissues, including the liver (Rock *et al.* 1996, Clinton 1998, Rao and Agarwal 1999).

Besides tocopherols, carotenoids are another group of antioxidant nutrients assumed to exert beneficial effects on health, including on glucose metabolism (Rock *et al.* 1996, Clinton 1998). Whether the antioxidant property of carotenoids is primarily responsible for putative beneficial effects has, however, been questioned (Pryor *et al.* 2000). Carotenoids also have other biological functions, including the ability to modulate gene expression and to enhance gap junction communication between cells (Rock *et al.* 1996, Stahl and Sies 1996, Rao and Agarwal 1999, Pryor *et al.* 2000). Furthermore, the effects of carotenoids may be mediated not only by the carotenoid itself but also via retinoid metabolites or other derivatives. For example, retinoic acid may, by acting through retinoid X receptors, influence cell differentiation and proliferation (Pryor *et al.* 2000).

Data on associations between dietary and plasma carotenoids and glucose metabolism are inconsistent. While the intake of β-carotene has been unrelated to GHb in cross-sectional studies (Shoff *et al.* 1993, Boeing *et al.* 2000), high dietary intake of β-cryptoxanthin was linked to a decreased risk of T2D in one cohort study (Montonen *et al.* 2004). A recent study by Wang *et al.* (2006), found no relationship between the intake of lycopene or the consumption of lycopene-rich tomato-based food products and the risk of T2D in women.

The serum concentrations of carotenoids (including α- and β-carotene, lycopene and/or lutein) were inversely related to fasting serum insulin concentration or insulin resistance in the cross-sectional studies of Ford *et al.* (1999), Facchini *et al.* (2000) and Coyne *et al.* (2005). Some of the carotenoids also showed inverse associations with fasting and/or 2-h glucose

levels and with the degree of glucose intolerance (Ford *et al.* 1999, Coyne *et al.* 2005). In a cross-sectional study among subjects without a diagnosis of diabetes, plasma lycopene concentration showed an inverse association with GHb ($P = 0.06$) (Bates *et al.* 2004). Serum β-carotene was not associated with the risk of T2D independent of cardiovascular risk factors (Reunanen *et al.* 1998), and also supplementation with β-carotene (50 mg on alternate days) for 12 years was unrelated to T2D risk in men (Liu *et al.* 1999). Moreover, in the study by Czernichov *et al.* (2006), supplementation with antioxidant vitamins and minerals including 6 mg of β-carotene for 7.5 years had no effect on fasting plasma glucose, although the baseline dietary and plasma concentrations of β-carotene were inversely associated with the outcome variable.

To summarize, results concerning associations of tocopherols and carotenoids with glucose metabolism are inconsistent. Reasons for discrepancies are unclear, although characteristics of the study populations, such as genetic heterogeneity, could have played a role. Therefore, associations between antioxidative nutrients and glucose metabolism should be studied in subjects at high risk for T2D. In these subjects, antioxidant intake may play a special role due to the underlying abnormalities in glucose metabolism precipitating oxidative stress and predisposing to T2D.

2.3.3.3 Dietary fibre

Dietary fibre refers to edible parts of dietary components that are resistant to digestion and absorption in the human small intestine, but nevertheless exert diverse physiological effects with demonstrable health influences (Ha *et al.* 2000, American Association of Cereal Chemists 2001). Agreement about the exact definition of dietary fibre has not been reached (Food and Agriculture Organization 1998), but according to the definition by the American Association of Cereal Chemists (2001), it includes polysaccharides, oligosaccharides, lignin and associated plant substances.

A traditional way to classify dietary fibre has been as soluble and insoluble. Vegetables, legumes, fruits and berries are a good source of soluble fibre, which reduces the rate of glucose absorption in the small intestine and thus attenuates postprandial blood glucose response (Jenkins *et al.* 2000), possibly via increased skeletal muscle plasma membrane GLUT4 content (Song *et al.* 2000). Whole-grain products are rich in insoluble fibre, which is non-viscous and has no effect on glucose absorption, but nevertheless has been associated with increased insulin sensitivity; though possible mechanisms have remained obscure (Jenkins *et al.* 2000). Further mechanisms by which dietary fibre could affect glucose metabolism include decreased inflammation (Ma *et al.* 2006) as well as increased intestinal proglucagon gene expression and secretion of proglucagon-derived peptides, such as glucagon-like peptide-1, with ensuing reduction in gastric emptying rates, enhancement of glucose uptake and disposal in peripheral tissues, reduced

HGP and effects on insulin secretion (Reimer *et al*. 1997, Massimino *et al*. 1998, American Dietetic Association 2002, Holst and Gromada 2004). Furthermore, high-fibre diets lead to increased production of short-chain fatty acids by intestinal bacteria, which might through increased hepatic glucose oxidation, increased hepatic insulin clearance and decreased NEFA release improve insulin sensitivity (McKeown and Mayer 2004). Finally, fibre can be a marker for other components of fibre-rich foods, *e.g*., magnesium and thiamine, which may be partially responsible for the beneficial effects (Bakker *et al*. 1998, Venn and Mann 2004).

Since the proposal of Trowell (1975) about a role of dietary fibre in the aetiology of diabetes, several epidemiological and experimental studies have explored the topic. Results available up to the time of gathering of the present dietary data were concluded to support a beneficial role for soluble but not necessarily for insoluble fibre in glucose metabolism (Feskens 1992). Since then, several studies have, however, found an inverse association between the intake of insoluble cereal fibre and future T2D (Salmerón *et al*. 1997a, b, Stevens *et al*. 2002, Montonen *et al*. 2003, Schulze *et al*. 2004; reviewed by Parillo and Riccardi 2004 and Montonen 2005). The overall inference has recently been that increased intake of (insoluble) dietary fibre is associated with beneficial effects on insulin sensitivity and protects against the development of T2D (Bessesen 2001, World Health Organization 2003, Parillo and Riccardi 2004, Steyn *et al*. 2004, Venn and Mann 2004, Lindström 2006). The topic has not, however, been studied in relatives of patients with T2D.

Summary

To conclude, the prevailing literature on associations between glucose metabolism and fatty acids, tocopherols, carotenoids (and dietary fibre) is inconsistent. One underlying factor behind the discrepant results may be the genetic heterogeneity of the study populations. Although the presence of a genetic component contributing to the individual metabolic response to dietary intake has been assumed for quite a while (Holtzman 1988), it was not until recent years that nutrient-gene interaction studies (referred to as 'nutritional genetics' or 'nutrigenetics' studies) have begun to reveal data on the different responsiveness among individuals to the same diet due to genetic variations (Gillies 2003, German 2005). Investigating the associations in high-risk populations and exploring possible nutrient-gene interactions are, however, essential for improving the understanding of T2D aetiology (Saltiel and Kahn 2001). Ideally, this information would help to develop prevention programmes that take into account the genetic predisposition to the disease.

3 Aims of the Study

This study was undertaken to investigate in non-diabetic relatives of patients with T2D cross-sectional associations of the following dietary factors with insulin resistance, β-cell function and/or basal and post-load glucose and NEFA levels:

1. Total fat intake and dietary and plasma cholesterol ester fatty acids (I and II). Possible interactions of fatty acids with the *PPARG* Pro12Ala polymorphism on the outcome variables were taken into consideration.

2. Dietary and plasma carotenoids (α- and β-carotene and lycopene) and α- and γ-tocopherol (III).

3. Total, water-insoluble and water-soluble dietary fibre (IV).

4 Subjects and Methods

4.1 Subjects and study design

The Botnia Dietary Study investigates relationships of dietary factors to measures of glucose metabolism in relatives of patients with T2D, comprising a high-risk group for the disease. The gathering of the dietary data occurred in conjunction with the follow-up investigation of the Botnia Study, which is a prospective family study on genetic and metabolic defects of T2D initiated along the western coast of Finland in 1990 (Groop *et al*. 1996, Tripathy *et al*. 2000). At the beginning of the main Botnia Study, all patients with known T2D from four health care centres (Jakobstad, Korsholm, Malax-Korsnäs and Närpes), their family members and spouses without a family history of diabetes were invited to participate. The study includes the baseline investigation and follow-up examinations. The Botnia Study protocol was approved by the local ethics committees.

The Botnia Dietary Study was initiated in October 1994. The inclusion criteria of the dietary study were as follows: 1) a first-degree (including parents, children and siblings) or second-degree (including grandparents, grandchildren, aunts, uncles and children of siblings) family history of T2D, 2) an age of 20-70 years at the time of the Botnia baseline investigation and 3) normal glucose tolerance (NGT) or IGT according to the 1985 WHO criteria (World Health Organization 1985). Family history of T2D was determined from hospital records or by an OGTT (Groop *et al*. 1996).

The formation of the study population is depicted in Figure 2. By June 1997, a consecutive sample of 746 subjects who fulfilled the inclusion criteria had been invited to participate. Of those invited, 603 (81%) agreed to participate and completed the first 3-day food record acceptably (two subjects were excluded because of incomplete recording done after the assigned study days). Two 3-day food records were completed by 590 subjects (79%). Fourteen subjects with newly diagnosed diabetes according to the latest WHO criteria (Alberti and Zimmet 1998) at the time of the dietary study were excluded from the analyses. Exclusions because of missing data and other criteria are detailed in the original publications.

4.2 Measurement of dietary intake

Dietary intake was measured by means of two 3-day estimated food records on average 6 months (range 5-11) apart. In Studies I, II and III, average food consumption and nutrient intakes were determined from the first 3-day period only, as in these studies data on plasma levels of nutrients were used as well, and plasma data were available only from the time of the first recording period. In Study IV, dietary intake data from both 3-day food records were combined.

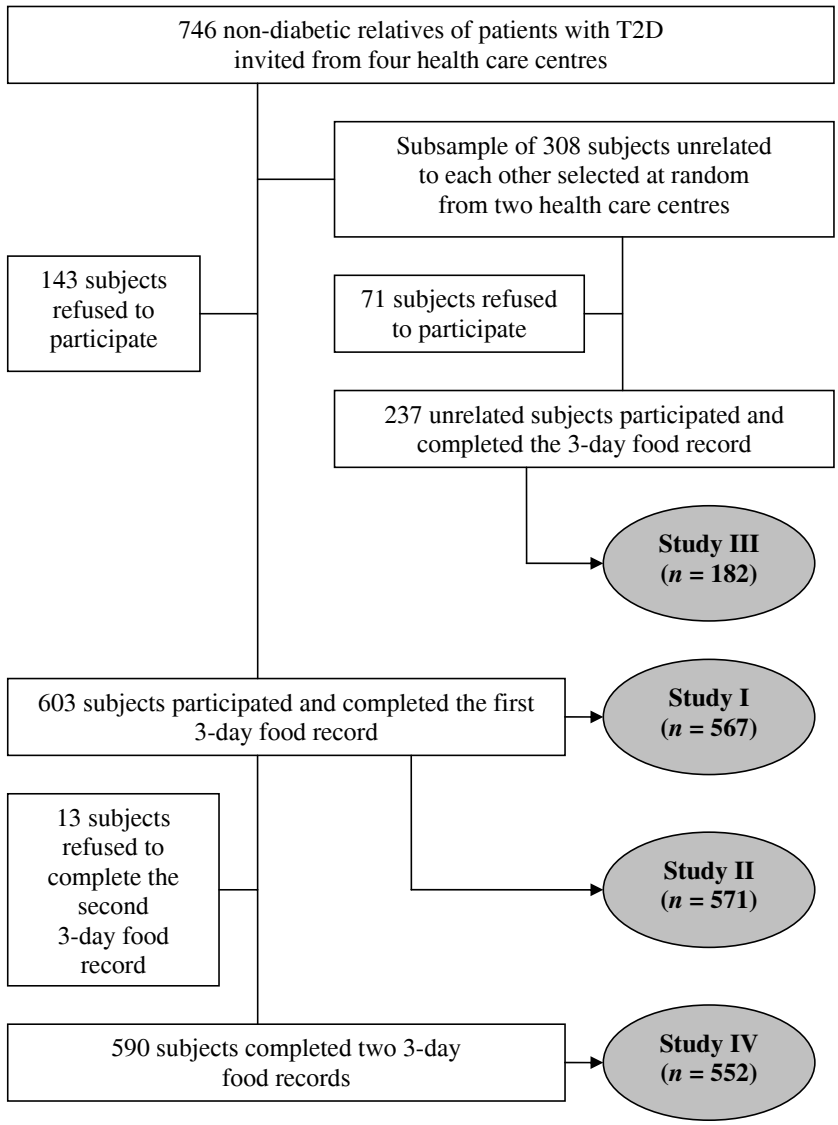

Figure 2. Formation of the study populations. Exclusions because of missing data and other criteria are detailed in the original publications

The protocol of the Botnia Dietary Study included altogether three visits to the local health care centre. During the first visit, the purpose of the dietary study was explained to subjects fulfilling the inclusion criteria and their willingness to participate was ascertained. This visit included a medical examination, including a standard OGTT and a structured interview on demographic data performed by trained research staff. During the second visit, which occurred as soon as possible after the recording period, the food record was checked, and in two of the health care centres an IVGTT was performed. For the second recording period after about 6 months, the food record form was sent to participants, and after the recording, participants visited the health care centre once more to return the form.

The field work of the dietary study was undertaken by local research staff after completing an intensive training programme during which they were taught how to keep a food record, how to instruct the participants about the recording and how to check the record. The collection of dietary data was overseen by the primary researcher (KY) throughout the study period.

The subjects who agreed to participate in the dietary study were given a food record form and requested to complete it over a 3-day consecutive period, detailing all foods and drinks consumed. Recording days were assigned by the research staff, and each 3-day period included two weekdays and one day of the weekend. Detailed verbal instructions on filling in a food record were given individually, but the recording form also included written instructions. Participants were advised to record every item of food or drink consumed in as detailed a manner as possible immediately after each eating episode. Information on food preparation methods and brand names and details of individual ingredients of composite dishes were requested. It was emphasized that it was very important not to change customary food habits, *i.e.*, the amount or type of the food or drink consumed during the study period. The amount of food and drink was estimated using a booklet of food photographs with different portion sizes (Haapa *et al*. 1985) or with household measurement units. The figures of the portion size booklets were supplied with legends in Swedish, as most of the study subjects were Swedish-speaking. The intake of dietary supplements was also recorded.

After the recording period, the participants visited the health care centre and returned the completed food record, which was then reviewed by the research staff for recording deficiencies. Any ambiguities were resolved with the participant. After receiving the food records, the primary researcher (KY) reviewed them once more, and if missing information was evident, the research staff was asked to contact the participant by phone for completion of data.

Food intake data were entered into the computer by the researcher (KY) and by a student majoring in nutrition. The data were analysed using the software and the Finnish National Food Composition Database developed and maintained at the National Public Health Institute, Helsinki, Finland

(Ovaskainen *et al.* 1996). The nutritional information of the database is based on Finnish food analyses and international food composition tables. To maximize the accuracy of the nutrient intake data, recipes and ingredients specified in the food records were input for analysis. In addition, local bread, commonly consumed by study participants, was added to the nutrient database using the manufacturer's information on the food label. Data on the content of dietary supplements were obtained from the National Food Administration and from manufacturers.

4.3 Medical examination

The participants visited the local health care centre in the morning after an overnight fast. During the medical screening, weight, height, waist and hip circumferences and blood pressure were measured, samples for the measurements of serum lipids, plasma cholesterol ester fatty acids, carotenoids and tocopherols drawn, an OGTT performed, and data on demographic characteristics obtained.

During a standardized 75-g OGTT, blood samples for the measurement of plasma glucose and serum insulin were drawn at -5, 0, 30 and 120 min, and for serum NEFA at 0 and 120 min. During the second visit, after completion of the first 3-day food record, subjects attending the health care centres in Malax-Korsnäs and Närpes underwent an IVGTT, in which a bolus of glucose (300 mg/kg body weight in a 50% [by vol] solution) was administered intravenously within 2 min. Samples for the measurement of serum insulin were drawn at -10, -5, 0, 2, 4, 6, 8 and 10 min.

For anthropometric measures, the subjects were standing and dressed in light indoor clothing without shoes. Body weight and height were measured, and BMI was used as the measure of relative body weight. Waist circumference was measured with a soft tape midway between the lowest rib and the iliac crest, and hip circumference over the widest part of the gluteal region. The waist-to-hip ratio (WHR) was used as the measure of central adiposity. Blood pressure was measured after 30 min of rest from the right arm with the subject seated. The average of the two readings was used.

4.4 Structured questionnaire on demographic data and physical activity

Data on smoking, education and physical activity (Mälkiä *et al.* 1988) were obtained by a structured questionnaire. The level of education was defined as the total number of years of school and professional education.

Data on physical activity during the last 12 months were changed to metabolic equivalents (METs), which represent an estimate of the relative

intensity of activity and describe the amount of energy needed during exercise compared with resting energy expenditure (1 MET is approximately equal to an energy expenditure of 1 kcal [4.2 kJ]/kg of body weight per hour). Physical activity at work was reported according to a seven-point scale, ranging from no work and no activity (MET = 1.5) to very heavy manual work (MET = 10.0). Exercise while travelling to work was classified by tertiles: the first tertile represented travel by motor vehicle (MET = 1.5), the second by walking (MET = 3.5) and the third by bicycle (MET = 5.0). Leisure time exercise received MET values from 2.0 to 12.5 depending on the type and intensity of the activity. The sum of MET values during work, the work trip and leisure time described the total physical activity and was used in statistical analyses.

4.5 Measurement of glucose metabolism

The main outcome variables used to describe glucose metabolism were insulin resistance, β-cell function, and fasting and 2-h post-load (OGTT) plasma glucose concentrations. Fasting and 2-h serum NEFA levels during the OGTT were used as additional outcome variables.

Insulin resistance was determined by the homeostasis model assessment - insulin resistance index (HOMA-IR), which estimates insulin sensitivity from fasting plasma glucose and fasting serum insulin concentrations (Matthews *et al*. 1985). Low HOMA-IR values indicate low insulin resistance (and high insulin sensitivity), whereas high HOMA-IR values indicate high insulin resistance (and low insulin sensitivity).

Beta-cell function was described by two variables. The insulinogenic index (IGI) was calculated as the ratio of the increment in serum insulin concentration at 30 min to the increment in plasma glucose concentration at 30 min during the OGTT ($[Ins_{30}\text{-}Ins_0]$ / $[Glu_{30}\text{-}Glu_0]$) (subjects with a 30-min glucose concentration less than or equal to the fasting glucose concentration were excluded from the analyses concerning this outcome variable) (Phillips *et al*. 1994). To describe first-phase insulin secretion during an IVGTT, the incremental area under the insulin curve during the first 10 min of the IVGTT was calculated. Low values of these variables are markers of first-phase β-cell dysfunction.

Fasting and 2-h glucose concentration values during the OGTT were used as indicators of glucose tolerance. Glucose tolerance status (NGT, IFG, IGT or diabetes) at the time of the dietary study was determined according to the latest WHO criteria (Alberti and Zimmet 1998).

4.6 Assays

Glucose, insulin, NEFA and lipids. Plasma glucose concentrations were measured with a glucose oxidase method (Beckman Glucose Analyser II, Beckman Instruments, Fullerton, CA). Serum insulin concentrations were measured by radioimmunoassay (Pharmacia, Uppsala, Sweden), with an interassay coefficient of variation < 9%. Serum NEFA concentrations were measured by an enzymatic colorimetric method using a commercially available kit (Wako Chemicals GmbH, Neuss, Germany). From fasting venous samples, serum TG and total and high-density lipoprotein (HDL) cholesterol concentrations were measured by enzymatic methods using the Cobas Mira autoanalyser (Hoffman-La Roche, Basel, Switzerland). Serum HDL cholesterol concentration was measured enzymatically after precipitation of the apoB-containing lipoproteins with heparin and manganese chloride. Serum low-density lipoprotein (LDL) cholesterol concentration was calculated from fasting total cholesterol, TG and HDL cholesterol values using the Friedewald formula (Friedewald *et al*. 1972) if the serum TG concentration did not exceed 5 mmol/l.

Genotyping. The PPAR-*γ*2-exon 6 Pro12Ala polymorphism was genotyped by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method and agarose gel electrophoresis using primers 5'- GAT AGA GAC AAA ATA TCA GTG (forward) and 5'- GTA TCA GTG AAG GAA TCG CTT TCC G (reverse). PCR was carried out in a 20-µl volume containing 5XNH4-buffer, 10 µmol/l of each dNTP, 3 mmol/l $MgCl_2$, 0.5 U Taq polymerase (Amersham Pharmacia Biotech), 1.5% formamide, 4 pmol of each primer and 25 ng of genomic DNA. The cycling conditions were 96°C for 3 min, 38 cycles of 96°C for 30 s, 56°C for 30 s and 72°C for 60 s, followed by a final extension at 72°C for 10 min. PCR products were followed by digestion with BstU-1 at 60°C for 2 h and separated on 6% agarose gel (SeaKem) stained with ethidium bromide. The common allele 1 (*Pro12*) gave a 113-bp fragment and the variant allele 2 (*Ala12*) 87-bp and 25-bp fragments.

Plasma cholesterol ester fatty acid composition. For plasma cholesterol ester fatty acid determinations, a fasting sample was drawn and initially stored for 1-3 months at -20°C and thereafter at -70°C until analysed. Serum fat was extracted from plasma with dichoromethane-methanol (Folch *et al*. 1957), cholesterol esters separated by thin-layer chromatography (Merck silica gel G 0.25 mm) and methylated with acidic methanol (5% H_2SO_4) at 85°C for 2 h to produce methyl esters (Stoffel *et al*. 1959). The percentage composition of the sample was determined with an HRGC 412 Micromat gas chromatograph (HNU Nordion Instruments, Helsinki, Finland) equipped with a 25-m-long NB-351 column (I.D. 0.32 mm, phase layer 0.20 µm, HNU-Nordion Instruments), split injection, helium as carrier gas in a temperature program from 180°C to 230°C. Peaks from 14:0

to 22:6*n*–3 were identified and the percentage composition calculated with a Sunicom Workstation (Helsinki, Finland). The composition of identified fatty acids was normalized to 100%. Between-series variability was 1-3% for peaks over 4% and 5-10% for smaller peaks (for docosahexaenoic acid [DHA] 35%).

Plasma tocopherols and carotenoids. Plasma concentrations of α- and γ-tocopherol, α- and β-carotene and lycopene were analysed from the same fasting samples as plasma cholesterol ester fatty acid composition. To 0.2 ml of plasma, tocol and echinenon as internal standards for tocopherols and carotenoids, respectively, and a solution (50% [by vol] ethanol) containing 1% (by vol) ascorbic acid were added. After extraction with hexane, the tocopherols (Anttolainen *et al.* 1996) and carotenoids (Bieri *et al.* 1985) were analysed separately by high-performance liquid chromatography. Ratios of peak height to internal standard were compared with those of reference plasma for which concentration values were traceable to certified serum standards (standard reference material 968b; National Institute of Standards and Technology, Gaithersburg, MD). The precision between series was 5.7% for tocopherols and 7.9% for carotenoids.

4.7 Statistical analyses

Statistical analyses were performed with the statistical packages BMDP (version 7.1; BMDP Statistical Software Inc., Los Angeles, CA) and SAS (versions 8.1 and 8.2; SAS Institute Inc., Cary, NC) for UNIX. The criteria for statistical significance as two-sided *P* values are defined in the original communications. Descriptive data were presented as mean (SD), *n* (%) or median (25^{th} and 75^{th} percentiles).

The normality of variables was determined with the Shapiro-Wilk *W* test. A logarithmic, square root or reciprocal transformation was applied for variables with skewed distributions if a better approximation to normal distribution was obtained. When near-normality was not achieved, non-parametric tests were applied or the variables were treated as categorical in the analyses.

The significance of differences between groups in continuous variables was analysed with the Student's unpaired *t* test, the Mann-Whitney *U* test or the analysis of covariance. The significance of differences in categorical variables was tested with χ^2 test. Partial Pearson's product-moment correlation and Spearman's rank correlation tests were used to examine adjusted interrelationships between variables of interest.

Associations of dietary and plasma exposure variables with outcome measures of glucose metabolism were evaluated with multiple linear regression analysis. Since in Studies I, II and IV the participants could be relatives, the analyses were performed with the PROC MIXED procedure

(SAS/STAT software; SAS Institute Inc., Cary, NC), where the family connection was considered. Potential interaction with the *PPARG* Pro12Ala polymorphism in Studies I and II was explored in models that included main effects and the product term of the genotype and the exposure variable, and thereafter in genotype-stratified analyses. A statistical interaction exists when the degree or direction of the association of a dietary variable with an outcome variable differs according to the genotype. If there was no indication of interaction, the interaction variable and the genotype variable were omitted from the model, and results were presented as such. To evaluate whether associations between exposure and outcome variables were independent of potential confounding factors, these were added to the models. Potential confounding factors were identified from earlier reports and from correlation analyses of the present studies.

In regression models, dietary fatty acids were expressed as proportions of total fatty acids similarly as plasma cholesterol ester fatty acids (Studies I and II). Otherwise, the intakes of nutrients were adjusted for energy intake according to the residual adjustment method (Willett and Stampfer 1986). Measures of glucose metabolism, dietary, clinical, anthropometric and demographic variables, except sex and smoking, were included in the models primarily as continuous variables. Exceptions are outlined in the original publications.

5 RESULTS

Dietary and plasma cholesterol ester fatty acids in relation to glucose metabolism (Studies I and II)

Several statistically significant differences were observed between men and women in the intakes and plasma levels of fatty acids (Study I, Table 1 and Study II, Table 1). Therefore, the analyses concerning fatty acids were performed mainly separately for men and women.

The proportions of *Pro/Pro*, *Pro/Ala* and *Ala/Ala* genotypes were 75.7%, 22.0% and 2.3%, respectively. The distribution of the genotypes did not significantly deviate from Hardy-Weinberg predictions ($P > 0.05$). Because of the low number of *Ala/Ala* subjects (5 men and 8 women), *Pro/Ala* and *Ala/Ala* subjects were considered one group and compared with *Pro/Pro* subjects in the regression models studying interaction between the *PPARG* Pro12Ala polymorphism and the fatty acid exposure variables. The proportion of plasma EPA was higher in *Ala* allele carrier women than in homozygote *Pro/Pro* women (1.67% *vs*. 1.44%, $P = 0.006$).

In men, regression models studying interaction between the *PPARG* Pro12Ala polymorphism and dietary and plasma fatty acids showed a significant interaction for the intake of γ-linolenic acid ($P = 0.001$) and for plasma stearic acid ($P = 0.002$) on the 2-h plasma glucose concentration. In the genotype-stratified analyses, high intake of γ-linolenic acid (regression coefficient [SE] -1.17 [0.28], $P < 0.001$) and high plasma stearic acid proportion (regression coefficient [SE] -0.23 [0.08], $P = 0.010$) were associated with low 2-h plasma glucose in *Pro/Pro* men, whereas no association was seen in *Ala* allele carrier men ($P = 0.12$ and $P = 0.22$, respectively) (Study I, Table 3).

In women, a significant interaction ($P = 0.007$) between the *PPARG* Pro12Ala polymorphism and dietary palmitic acid on the fasting plasma glucose concentration was observed. In the stratified analyses, palmitic acid was directly related to the outcome variable in *Ala* allele carrier women (regression coefficient [SE] 0.02 [0.005], $P = 0.015$) but not in *Pro/Pro* women ($P = 0.42$) (Study I, Table 3). Furthermore, in women, a significant interaction ($P = 0.008$) was observed between the *PPARG* Pro12Ala polymorphism and dietary arachidonic acid on 2-h glucose levels, although in the stratified analyses, the opposite associations in the genotype groups (direct in the *Pro/Pro* homozygous and inverse in the *Ala* allele carriers) did not reach significance ($P = 0.093$ and $P = 0.064$, respectively) (Study I, Table 3).

Regarding marine *n*–3 fatty acids, a significant interaction ($P = 0.036$) was present between the *PPARG* Pro12Ala polymorphism and plasma DHA on the fasting NEFA concentration in men. The genotype-stratified models showed an inverse association in *Pro/Pro* homozygotes (regression coefficient [SE] -0.14 [0.06], $P = 0.028$), but not in *Ala* allele carriers ($P = 0.40$) (Study II, Table 2). In the stratified models, also the intakes of fish (regression coefficient [SE] -0.13 [0.05], $P = 0.015$), EPA (regression coefficient [SE] -0.05 [0.02], $P = 0.022$) and DHA (regression coefficient [SE] -0.05 [0.02], $P = 0.023$) showed inverse associations with this outcome variable in *Pro/Pro* men, but not in *Ala* allele carrier men ($P \geq 0.45$) (*P* for interaction between these exposure variables and the polymorphism was 0.10-0.12).

A significant interaction between the *PPARG* Pro12Ala polymorphism and plasma EPA on insulin resistance was observed in men ($P = 0.040$; in women *P* for interaction was 0.12) (Study II). When men and women were analysed together in the stratified model, a high proportion of plasma EPA was associated with low insulin resistance in *Ala* allele carriers (regression coefficient [SE] -0.17 [0.08], $P = 0.038$), while no association was seen in the *Pro/Pro* genotype group (regression coefficient [SE] 0.04 [0.04], $P = 0.28$).

A significant interaction ($P = 0.020$) between the *PPARG* Pro12Ala polymorphism and fish intake on 2-h glucose concentration was found in women. Genotype-stratified models showed an inverse association in *Ala* allele carriers (regression coefficient [SE] -0.14 [0.05], $P = 0.039$), but not in *Pro/Pro* homozygotes ($P = 0.87$) (Study II, Table 3).

Regarding the plasma surrogates of elongase and desaturase enzymes, in women a significant interaction was observed between the *PPARG* Pro12Ala polymorphism and Δ5-desaturase on insulin resistance ($P = 0.006$) and on the 2-h plasma glucose concentration ($P = 0.005$). In the genotype-stratified models, inverse associations were observed in *Ala* allele carrier women (for insulin resistance regression coefficient [SE] -0.72 [0.21], $P = 0.011$, and for the 2-h plasma glucose regression coefficient [SE] -0.57 [0.13], $P = 0.003$), but not in *Pro/Pro* homozygotes ($P = 0.40$ and $P = 0.62$, respectively) (Study I, Table 3). In men, a significant interaction ($P = 0.007$) with the *PPARG* polymorphism was found for Δ5-desaturase on the fasting plasma glucose, but the opposite associations in the genotype groups (direct in *Pro/Pro* homozygous and inverse in *Ala* allele carriers) were not significant in the stratified models ($P = 0.14$ and $P = 0.16$, respectively) (Study I, Table 3).

A summary of the fatty acids and the surrogates of desaturase enzymes for which the present findings found an interaction with the *PPARG* Pro12Ala polymorphism on measures of glucose and NEFA metabolism separately in men and women is presented in Table 1. As the results suggested that several of the interactions might be sex-specific, formal tests

for interactions between sex, genotype and exposure variable on the outcomes were performed. These analyses yielded the following *P* values for the third-order interaction terms (sex * genotype * exposure): dietary palmitic acid on fasting plasma glucose, $P = 0.002$; dietary γ-linolenic acid on 2-h plasma glucose, $P = 0.054$; dietary arachidonic acid on 2-h plasma glucose, $P = 0.034$; dietary EPA on fasting serum NEFA, $P = 0.078$; dietary DHA on fasting serum NEFA, $P = 0.055$, and on 2-h plasma glucose, $P = 0.15$; plasma stearic acid on 2-h plasma glucose, $P = 0.14$; plasma EPA on insulin resistance, $P = 0.26$, and on 2-h plasma glucose, $P = 0.49$; plasma DHA on fasting serum NEFA, $P = 0.049$, and on 2-h plasma glucose, $P = 0.019$; Δ5-desaturase on fasting plasma glucose, $P = 0.28$, on insulin resistance, $P = 0.23$, and on 2-h plasma glucose, $P = 0.71$. Thus, according to the present findings, an interaction between the Pro12Ala polymorphism and dietary palmitic acid on the fasting plasma glucose is sex-specific, as is that of dietary arachidonic acid on the 2-h plasma glucose and those of plasma DHA on the fasting serum NEFA and on the 2-h plasma glucose.

Table 1. Fatty acids and the surrogates of desaturase enzymes for which the present findings suggest an interaction with the *PPARG* Pro12Ala polymorphism on measures of glucose and NEFA metabolism.

	HOMA insulin resistance	**Fasting plasma glucose**	**2-h plasma glucose**	**Fasting serum NEFA**
Men				
Dietary γ-linolenic acid			×	
Dietary EPA and DHA				×
Plasma stearic acid			×	
Plasma EPA	×			
Plasma DHA				×
Δ5-desaturase		×		
Women				
Dietary palmitic acid		×		
Dietary arachidonic acid			×	
Dietary DHA			×	
Plasma EPA	×		×	
Plasma DHA			×	
Δ5-desaturase	×		×	

In addition to the above-mentioned genotype-specific associations, high plasma dihomo-γ-linolenic acid was associated with high insulin resistance (regression coefficient [SE] 0.28 [0.10], $P = 0.006$) and with high 2-h plasma glucose concentration (regression coefficient [SE] 0.17 [0.06], $P = 0.007$) in the combined group of women (*P* for interaction with the *PPARG* polymorphism ≥ 0.20) (Study I).

Furthermore, the plasma proportion of DHA was inversely associated with insulin resistance in all men (regression coefficient [SE] -0.13 [0.06], $P = 0.035$), and the intakes of EPA (regression coefficient [SE] -0.04 [0.01], $P = 0.006$) and DHA (regression coefficient [SE] -0.04 [0.01], $P = 0.015$) were inversely associated with 2-h serum NEFAs in all women.

Dietary and plasma tocopherols and carotenoids in relation to glucose metabolism (Study III)

The dietary intake of α-tocopherol was inversely associated with the fasting plasma glucose concentration in women (regression coefficient [SE] -0.54 [0.25], $P = 0.032$) (Study III, Table 5). In contrast, a direct association between plasma α-tocopherol concentration and 2-h plasma glucose levels was observed in both men (regression coefficient [SE] 0.39 [0.18], $P = 0.038$) and women (regression coefficient [SE] 0.42 [0.16], $P = 0.013$).

An inverse association between the intakes of α-carotene (regression coefficient [SE] -0.07 [0.02], $P = 0.005$), β-carotene (regression coefficient [SE] -0.24 [0.08], $P = 0.006$) and lycopene (regression coefficient [SE] -0.60 [0.14], $P < 0.0001$) and the fasting plasma glucose concentration was observed in men (Study III, Table 5). In addition, plasma β-carotene was inversely related to insulin resistance in men (regression coefficient [SE] -0.28 [0.09], $P = 0.003$). In women, on the other hand, plasma β-carotene showed a direct association with the fasting plasma glucose concentration (regression coefficient [SE] 0.25 [0.11], $P = 0.026$) (Study III, Table 5).

Dietary fibre in relation to glucose metabolism (Study IV)

The intakes of total (regression coefficient [SE] -0.17 [0.07], $P = 0.012$), insoluble (regression coefficient [SE] -0.15 [0.07], $P = 0.024$) and soluble fibre (regression coefficient [SE] -0.14 [0.07], $P = 0.049$) were inversely related to insulin resistance in men and women combined (Study IV, Table 3).

6 Discussion

6.1 Subjects and methods

Study population and study design

The present results should be interpreted in the light of the study population and the study design. The study subjects comprised a high-risk group for T2D because they were all first- (98%) or second-degree (2%) relatives of patients with diagnosed T2D. The subjects were thus not representative of the general population, and the findings are not generalizable beyond genetically susceptible persons.

The rationale for studying non-diabetic relatives of patients with T2D was to explore a high-risk population in which, because of genetically determined defects in glucose metabolism, the associations of dietary factors with glucose metabolism might be altered compared with the general population. Subjects at high risk for T2D are characterized by many metabolic alterations, such as abdominal obesity, insulin resistance, impaired β-cell function, reduced muscle glycogen synthesis, elevated fasting NEFA levels, increased intramyocellular lipid accumulation and endothelial dysfunction (Vaag *et al*. 1995, Groop *et al*. 1996, Kosaka *et al*. 1996, Perseghin *et al*. 1996, 1997, 1999, Balletshofer *et al*. 2000, Shulman 2000, Kashyap *et al*. 2003, Petersen *et al*. 2004). Because of these prediabetic derangements, the associations of nutritional factors with glucose metabolism could well be different from those in subjects with a different degree of genetic predisposition to the disease. Clues about differences in metabolic response between subjects with and without a family history of the disease have been observed regarding the effects of physical activity (Higgins *et al*. 2005). By studying relatives of patients with T2D, genetic heterogeneity among subjects, which might impede the finding of relevant associations, can be reduced. Studying subjects with inherited susceptibility but without manifest T2D is necessary when searching for associations between dietary factors and metabolic defects present early in the course of the disease.

The participation rate in the study was good (81%), but a response bias according to the health status of the subjects was evident. Those who chose not to participate had higher glucose and insulin levels, although in other features (age, BMI, WHR, physical activity, length of education) the refusers and the participants did not differ. Some caution is, therefore, necessary when interpreting the results and generalizing them to the original study population. Had the associations of dietary factors with

measures of glucose metabolism been markedly different between subjects who participated and those who did not, a bias would have been introduced.

The observational cross-sectional design of this study precludes inferences regarding causality. This is especially true when interpreting results concerning associations of plasma nutrient levels with measures of glucose metabolism, as disordered glucose metabolism may affect plasma nutrient levels (Arab 2003). However, regarding associations between dietary intake and, for example, insulin resistance, since the subjects were unaware of their insulin resistance status at the time of dietary data gathering, it is not unreasonable to assume that dietary factors affected insulin resistance and not *vice versa*.

Measurement of dietary intake

Accurate estimation of dietary intake is essential for establishing associations between nutritional factors and outcome variables (Marshall 2003). However, the assessment of usual diet is problematic due to large day-to-day and seasonal variation in food intake and misreporting (Tarasuk and Brooker 1997, Lissner *et al*. 1998).

The purpose of dietary data gathering here was to obtain representative data on habitual intake of the participants using an estimated food record. Data from 3 recording days were used in Studies I, II and III, and data from 6 days in Study IV. The strengths of food records include providing quantitative data and not relying on memory, but inconvenience of recording and socially desirable responding may lead to alteration of normal dietary habits. This may affect the type and quantity of foods and drinks consumed or reported (Thompson and Byers 1994, Macdiarmid and Blundell 1997). The fact that intake data based on 3-day food records do not give a very accurate picture of the usual diet due to high intra-individual variation in food consumption and nutrient intake is a major limitation of this study. Strengths include special attention being given to checking of food records and the nutrient database used being continuously updated and allowing special recipes to be input. Of special relevance to the present study was that the database comprises comprehensive data on fatty acids and enabled also the measurement of fatty acids of minute intake levels.

Self-reported food intakes are prone to underreporting leading to underestimation of energy and nutrient intakes (Lissner *et al*. 1998). Probability of underreporting is associated with obesity, female sex and older age (Heitmann and Lissner 1995, Hirvonen *et al*. 1997, Lissner *et al*. 1998, Livingstone and Black 2003). When studying obesity-related diseases, obesity-related underreporting is especially problematic, as it can lead to biased estimates of diet-disease relationships (Lissner *et al*. 1998). Selective overreporting of 'healthy' foods, such as vegetables and fruits, and underreporting of 'unhealthy' foods, such as cakes, sugar, candies and fats, may

lead to biased estimates of, *e.g.*, macronutrient intake (Heitmann and Lissner 1995, Tarasuk and Brooker 1997, Livingstone and Black 2003).

The relatively poor precision of dietary assessment distorts estimates of nutritional exposure. Often, it is likely to result in underestimates of exposure-outcome associations (Marshall 2003). Methods used to reduce the effects of underreporting include exclusion of subjects reporting low energy intake relative to energy requirements (Goldberg *et al.* 1991) and adjustment of nutrient intakes for energy intake (Willett and Stampfer 1986). Excluding underreporters from the data set may lead to substantial loss of subjects, thus reducing the statistical power of analyses and may also introduce an unknown bias if only a proportion of underreporters is identified (Macdiarmid and Blundell 1997, Lissner *et al.* 1998, Livingstone and Black 2003). Adjustment for energy intake reduces between-person variation in nutrient intake due to misreporting and allows all data to be included (Stallone *et al.* 1997, Willett *et al.* 1997, Lissner *et al.* 1998). This approach cannot, however, eliminate bias in nutrient intake if it is due to selective underreporting of, *e.g.*, high-fat foods. Although overall underreporting cannot be ruled out in the present data set, selective underreporting of fatty foods at group level seems improbable as the proportion of energy from fat was high in both men and women (37.0% and 35.8%, respectively) compared with statistics (33.4% in men and 31.5% in women) reported in the Finnish National Dietary Survey FINDIET 1997 (FINDIET Study Group 1998).

For the above reasons, all subjects were included in the analyses, and nutrient intake was mainly adjusted for energy intake according to the residual method of Willett and Stampfer (1986). In Studies I and II, both dietary and plasma fatty acids were, however, expressed as percentage of total fatty acids because expression in comparable units has been recommended (Connor 1996).

Biochemical indicators of dietary intake

The obvious limitations of self-reported dietary intake data have spurred the use of different biochemical indicators of dietary intake, also called 'biomarkers', in epidemiological studies (Katan 1998). These are more objective measures of the exposure, as they do not rely on capability to describe foods and are unaffected by social desirability issues (Marshall 2003, Potischman 2003). Biomarker levels are, however, not only influenced by dietary intake, but also by a number of other factors, including, but not limited to, absorption and metabolism of the nutrient, intake of other nutrients, age, sex, physical exercise, smoking, genetics and health status (Holman 1986, Blanck *et al.* 2003, Marshall 2003, Pollard *et al.* 2003, Potischman 2003).

The correlation coefficients between dietary and plasma fatty acids are usually relatively low, and opinions about the usefulness of dietary *vs.* biomarker fatty acid data differ (Potischman 2003, Vessby 2003). The low

correlations are certainly partly explained by uncertainties in dietary reporting, but probably also by plasma fatty acid levels being influenced by factors other than the intake of fatty acid *per se*. These include, *e.g.*, the relative amounts of other fatty acids, the metabolism of fatty acids in the body, nutritional status, genetic factors and disease processes (Zock *et al.* 1997, Arab 2003, Potischman 2003, Phinney 2005). Because two measures of an exposure can be mutually beneficial by providing a wider view of the exposure than only one measure (Connor 1996), both dietary intake and fatty acid composition of plasma were used in the analyses as fatty acid exposure variables.

The fatty acid composition of plasma cholesterol esters, which was used here, reflects the relative fatty acid intake during the past weeks (Ma *et al.* 1995, Zock *et al.* 1997). Significant correlations between dietary and plasma proportions were found for most SFAs and PUFAs, but not for oleic acid (Study I, Table 2), findings compatible with earlier data (Zock *et al.* 1997). In general, the correlation coefficients were lower in women. Whether this was due to dietary misreporting in women or reflects different handling or metabolism of fatty acids between the sexes (Luxon *et al.* 1998) is unclear. The finding of a higher proportion of DHA, but not eicosapentaenoic acid (EPA), in women than in men (Study II, Table 1) is in line with an earlier finding of higher cholesterol ester DHA in women (Giltay *et al.* 2004).

Plasma concentrations of α- and γ-tocopherol, α- and β-carotene and lycopene were used besides the intake of these nutrients as antioxidant exposure variables. A significant correlation between the dietary intake and plasma β-carotene was found only in women (Study III, Table 2). Earlier studies have reported significant correlations between dietary and serum β-carotene in both men and women (Wallström *et al.* 2001), only in men (El-Sohemy *et al.* 2002) and in neither gender (Carroll *et al.* 1999). The degradation of β-carotene has been suggested to be the cause of the weak correlations often observed (Jandacek 2000).

A significant correlation between plasma γ-tocopherol concentration and the intake of γ-tocopherol was found, while the plasma level of α-tocopherol was unrelated to its intake (Study III, Table 2). These findings are compatible with those of El-Sohemy *et al.* (2001), suggesting that plasma levels provide a better biomarker of γ-tocopherol intake than of α-tocopherol intake. Other previous studies have yielded mixed results about the correlation between dietary and blood α-tocopherol levels, as summarized by Ford and Sowell (1999).

Relationships between dietary intake and serum levels of antioxidants are thus not straightforward. Correlations may be diminished because of daily variation both in dietary intake and in blood concentrations (Wallström *et al.* 2001), but also due to oxidative stress if the nutrient is used as an antioxidant (Potischman 2003). Although some of the factors possibly confounding the relationships, such as smoking causing oxidative stress,

can be controlled statistically, others, *e.g.*, genetic differences in metabolism, may remain undetected, and thus, are not taken into consideration. Weak associations between dietary and serum antioxidants may thus indicate that serum levels are affected more by factors other than dietary intake and do not reflect differences in intake well. In the present study, this may apply to β-carotene in men and α-tocopherol in both sexes.

Measures of glucose metabolism

The outcome variables used to describe glucose metabolism were selected to represent the main metabolic features predictive of future T2D. They included insulin resistance, β-cell function and fasting and 2-h postload glucose and NEFA concentrations. The rationale for using several outcome variables was spurred by the assumption that different metabolic and genetic abnormalities of a heterogeneous disease, such as T2D, may characterize individuals (Matsuda and DeFronzo 1999). For example, in some subjects, the predominant defect may reside in the liver, whereas in others it may reside in muscle. While hepatic insulin resistance leads to excessive HGP and is manifested as fasting hyperglycaemia, insulin resistance in muscle results in postmeal glucose intolerance. The focus of our analyses was to evaluate associations of dietary factors with markers of these different metabolic defects.

The euglycaemic hyperinsulinaemic clamp is the standard reference method for the study of insulin resistance (Ferrannini and Mari 1998). In large study populations, estimates of insulin resistance are, however, usually obtained with cheaper and less time-consuming methods (Monzillo and Hamdy 2003). In the present study, insulin resistance was determined by the HOMA-IR, which estimates insulin resistance from fasting glucose and insulin concentrations (Matthews *et al.* 1985, Ferrannini and Mari 1998). This method has been regarded as a suitable surrogate of insulin resistance in subjects with euglycaemia and even with mild diabetes (Monzillo and Hamdy 2003), although according to some findings not necessarily in subjects with abnormal fasting glucose concentrations (Tripathy *et al.* 2004). While assumptions of this technique have raised some concern, and it has been postulated to describe primarily hepatic insulin resistance (Matsuda and DeFronzo 1999), estimates of insulin resistance based on HOMA-IR have correlated well with those obtained with the clamp technique (Matsuda and DeFronzo 1999, Monzillo and Hamdy 2003). A high HOMA-IR has also predicted the development of T2D (Haffner *et al.* 2000, Lyssenko *et al.* 2005).

Regarding β-cell function, first-phase insulin secretion was the main object of interest in the present study. The early insulin release plays an important role in the maintenance of glucose homeostasis, and its deterioration is predictive of future T2D (Lundgren *et al.* 1990, Lillioja *et al.* 1993, Haffner *et al.* 2000, Del Prato 2003, Lyssenko *et al.* 2005). The measures

used were insulin secretion during the first 10 min of an IVGTT and the insulinogenic index (IGI), which is based on glucose and insulin increments during the OGTT. Insulinogenic index has been shown to correlate well with first-phase insulin secretion in IVGTT in subjects with NGT and IGT (Phillips *et al.* 1994, Tripathy *et al.* 2004). Compared with estimates based on IVGTT, the IGI may provide even more physiological results, as it takes into account the gastrointestinal contribution to insulin secretion, which probably plays a role in the development of glucose intolerance (Holst and Gromada 2004). Of note, low values of the IGI have preceded the increase in fasting plasma glucose concentration (Kosaka *et al.* 1996) and have predicted the development of T2D (Haffner *et al.* 2000, Lyssenko *et al.* 2005).

High fasting and 2-h post-challenge glucose and NEFA concentrations have predicted the deterioration of glucose tolerance in several studies (Saad *et al.* 1991, Charles *et al.* 1997, Edelstein *et al.* 1997). In an analysis of six prospective studies, fasting and 2-h glucose values were the most important predictors of progression from IGT to T2D (Edelstein *et al.* 1997). Furthermore, fasting glucose concentrations even within the normoglycaemic range have been shown to be increased in the offspring of T2D parents compared with subjects without a family history of the disease, and have also predicted T2D in young men (Perseghin *et al.* 1999, Tirosh *et al.* 2005).

The glucose tolerance status of the subjects at the time of the dietary study was determined according to the latest WHO criteria based on an OGTT (Alberti and Zimmet 1998). Because of the day-to-day variation in the results of an OGTT, having only one test might result in misclassification (Alberti and Zimmet 1998). Therefore, the reproducibility of the classification of the subjects could have been improved if the OGTT had been repeated, but this was not feasible because of the large number of subjects in the Botnia Study.

6.2 Dietary factors in relation to glucose metabolism

The choice of the dietary factors studied was based on earlier data suggesting a role in glucose metabolism, but findings have been inconsistent. Fat amount and composition may be major contributors in the pathogenesis of T2D, but data on an interaction between fat and genetic factors determining glucose metabolism are scarce. Since α- and γ-tocopherol are the principal vitamin E compounds in human diets and plasma, and α- and β-carotene and lycopene are among the predominant carotenoids (Rock *et al.* 1996), these were selected for study.

6.2.1 Dietary and plasma cholesterol ester fatty acids

In our analyses, some of the associations observed between fatty acids and measures of glucose metabolism were dependent on the *PPARG* Pro12Ala polymorphism, while others were independent. Both of these findings were expected because some of the effects of fatty acids on glucose metabolism are likely to be mediated via PPAR-γ, as fatty acids are among its natural ligands, although fatty acids certainly also affect glucose metabolism independent of PPAR-γ. Obviously, an interaction between different polymorphisms and fatty acids is one thus far neglected factor behind the discrepancy in previous results concerning associations between fatty acids and glucose metabolism.

The analyses on associations between fatty acids and glucose metabolism were performed mainly separately for men and women because fatty acid metabolism may be dependent on sex hormones (Lopes *et al*. 1991). Many of the findings, indeed, turned out to be sex-specific. However, whether this was due to chance or methodological uncertainties or was a reflection of actual differences in response to exposure between sexes is unclear. Although sex differences in the results concerning dietary fatty acids could have been due to different dietary reporting between men and women, as social desirability issues have predicted underreporting only in women (Maurer *et al*. 2006), different results concerning plasma levels of fatty acids cannot be explained by sex-specific misreporting. Furthermore, the processes leading to impaired glucose metabolism may differ between men and women due to hormonal factors, sex dimorphism in the production of inflammatory cytokines and differences in the metabolic disturbances associated with insulin resistance (Wajchenberg 2000, Ding *et al*. 2006, van Genugten *et al*. 2006, North *et al*. 2007). For example, men may be less sensitive to the antilipolytic effects of insulin (Mittendorfer 2005), and the induction of insulin resistance by elevated plasma NEFAs may be restricted to men (Frias *et al*. 2001). Gene expression mediated by PPARs may also be dependent on sex (Khan and Vanden Heuvel 2003).

The size of the study population may be regarded as too small to evaluate any interaction between diet and genes. Therefore, in addition to the present findings, there may be other relevant interactions and *PPARG* genotype-specific associations between fatty acids and glucose metabolism. As regards the analyses in Study I, the *P* value was considered significant only when < 0.01 because of multiple testing of several fatty acids. Besides the interactions presented in Studies I and II, some interactions with a *P* value ≥ 0.01 were detected; some of these are mentioned below.

Plasma dihomo-γ-linolenic acid

Of earlier observations concerning associations between fatty acids and glucose metabolism, those of plasma dihomo-γ-linolenic acid with insulin resistance and glucose intolerance are among the most consistent and strongest (Vessby *et al.*1994a, b, Folsom *et al.* 1996, Lovejoy *et al.* 2001). Similarly, in the present analyses, high plasma dihomo-γ-linolenic acid was associated with high insulin resistance and plasma 2-h glucose levels, although only in women (Study I).

The dietary intake of dihomo-γ-linolenic acid is minimal, and its plasma level is dependent on formation from linoleic acid. Therefore, the associations of high plasma dihomo-γ-linolenic acid with worsened glucose metabolism have been suggested to be explained by the relationship of high plasma dihomo-γ-linolenic acid with low intake of linoleic acid, observed by Lasserre *et al.* (1985). According to this interpretation, low linoleic acid or high SFAs would be the real underlying factor leading to impairment of glucose metabolism (Vessby *et al.* 1994a). However, another explanation could be that high plasma dihomo-γ-linolenic acid levels are a result of the underlying disease process predisposing to T2D, and/or that dihomo-γ-linolenic acid *per se* or its eicosanoid derivatives adversely affect glucose metabolism. Dihomo-γ-linolenic acid is a precursor of the monoenoic prostaglandins (Stone *et al.* 1979), of which prostaglandin A1 was shown to induce endothelial apoptosis (Erl *et al.* 2004). This might contribute to insulin resistance via endothelial dysfunction (Pinkney *et al.* 1997), which has previously been observed to be an associate of disordered glucose metabolism (Hu and Stampfer 2003, Meigs *et al.* 2004).

As regards gene expression, a high level of activation was noted with prostaglandin A1 for PPAR-δ (Yu *et al.* 1995). Although PPAR-δ activation is associated with enhanced fatty acid burning in muscle (Grimaldi 2005), it has also been speculated that PPAR-δ overactivation might promote adipocyte phenotype in muscle, *e.g.*, via PPAR-γ activation (Jump and Clarke 1999, Holst *et al.* 2003). Abnormal accumulation of lipids in muscle, due to an imbalance between fatty acid uptake and rate of oxidation especially in subjects with subnormal oxidative capability, has been suggested to be the main pathogenic mechanism behind insulin resistance (Lewis *et al.* 2002, McGarry 2002). If dihomo-γ-linolenic acid or prostaglandin A1 leads to muscle PPAR-δ overactivation and thereby contributes to increased intramyocellular lipids, this might explain the common finding of a strong direct association between plasma dihomo-γ-linolenic acid level and disordered glucose metabolism.

Fatty acids with interaction with the PPARG Pro12Ala polymorphism

Dietary palmitic acid

A significant interaction between the *PPARG* Pro12Ala polymorphism and dietary palmitic acid on fasting plasma glucose was observed in women (Study I). In the genotype-stratified analyses, palmitic acid was directly related to the outcome variable in *Ala* allele carrier women, but not in *Pro/Pro* women. As palmitic acid is the major SFA in the diet, this finding is in line with the findings of Pisabarro *et al.* (2004), according to which a high intake of SFAs was associated with glucose intolerance only in *Ala* allele carriers. In the study of Luan *et al.* (2001), the ratio of dietary PUFAs to SFAs was inversely related to the fasting insulin concentration in *Ala* allele carriers, but not in *Pro/Pro* homozygotes. In accordance with this, in the present study, dietary palmitic acid was directly related to HOMA insulin resistance in *Ala* allele carrier women (regression coefficient [SE] 0.04 [0.02], $P = 0.032$), but not in *Pro/Pro* women (regression coefficient [SE] 0.01 [0.01], $P = 0.44$) (P for interaction > 0.05). The reasons for finding these associations in women only are at the moment unclear, but possibly related to hormonal factors. Also in the study of Robitaille *et al.* (2003), an interaction between the *PPARG* Pro12Ala polymorphism and dietary saturated fat was observed in women only in the sex-stratified analysis.

The prevailing notion concerning dietary fatty acid composition and glucose metabolism is that especially high intake of SFAs may be harmful (Lichtenstein and Schwab 2000, Vessby 2003, World Health Organization 2003, Steyn *et al.* 2004). Indeed, a direct relationship has been found between the intake of SFAs and increased insulin levels or insulin resistance (Maron *et al.* 1991, Parker *et al.* 1993, Feskens *et al.* 1994, Vitelli *et al.* 1996, Marshall *et al.* 1997), glucose levels or glucose intolerance (Feskens and Kromhout 1990, Salomaa *et al.* 1990, Færch *et al.* 2005) and development of T2D (Feskens *et al.* 1995). Moreover, in controlled intervention trials, with the most reliable study design, replacement of SFAs with PUFAs (Summers *et al.* 2002) or MUFAs (Vessby *et al.* 2001) has improved insulin sensitivity, although in the latter study only when the total intake of energy from fat was below the study population median. Furthermore, the fatty acid composition in serum lipids and skeletal muscle membranes typical of insulin resistance and diabetes has usually been high in palmitic acid (Salomaa *et al.* 1990, Vessby *et al.* 1994a, b). This association has been interpreted to be explained to a large extent by the fatty acid composition of the diet (Vessby 2003), although it is important to note that physical activity (Helge *et al.* 1999, Vessby *et al.* 2002), cardiorespiratory fitness (König *et al.* 2003), intrauterine and early nutrition (Baur *et al.* 1998, Ozanne *et al.* 1998), the background diet (Raatz *et al.* 2001), genetic factors (Storlien *et al.* 1996b), insulin resistance and endogenous synthesis and metabolism of fatty acids may also play a role (Vessby 2003).

In contrast to the above-mentioned results, in some epidemiological studies, the intake of SFAs has been unrelated to insulin (Sevak *et al*. 1994) and glucose levels (Mooy *et al*. 1995) as well as to the development of T2D (Marshall *et al*. 1994, Salmerón *et al*. 2001). In a recent analysis among the population of the Finnish Diabetes Prevention Study, saturated fat intake was not independently associated with the risk of T2D (Lindström 2006). The discrepancy in the literature concerning fatty acids and glucose metabolism has not received much attention, and possible underlying reasons seem to be unclear. Recent observations, including findings from the present study, suggest one explanation. Interaction between fatty acids and different polymorphisms related to glucose metabolism may play a role. Regarding the *PPARG* Pro12Ala polymorphism, it may be that a high intake of SFAs (or especially palmitic acid) is associated with impaired glucose metabolism (or especially high fasting plasma glucose) only in female *Ala* allele carriers; the genotype representing ~ 20% of the population (McCarthy 2004).

An explanation for the direct association between dietary palmitic acid and fasting glucose levels and insulin resistance in the *Ala* allele carrier but not in *Pro/Pro* women is still obscure. Among the effects of palmitic acid are induction of inflammation in adipocytes (Ajuwon and Spurlock 2005), decreased fat oxidation (Kien *et al*. 2005), reduction in the proliferative capacity of β-cells and induction of their death (Maedler *et al*. 2001) and repression of insulin gene expression in the presence of high glucose (Ritz-Laser *et al*. 1999). As especially the *Ala* allele carriers seem to be responsive to the amount of palmitic acid in the diet, it could be speculated that the high palmitic acid intake typical of Western diets might be an underlying explanation for the earlier observations of impaired insulin secretion characterizing particularly the *Ala* genotype (Mori *et al*. 2001, Stefan *et al*. 2001). Whether the effect could be mediated by reduced TG synthesis, channelling palmitic acid to pathways leading to apoptosis (Listenberger *et al*. 2003), is an interesting although at present only a speculative option.

Plasma stearic acid

In line with an earlier finding by Louheranta *et al*. (1998), the intake of stearic acid was unrelated to glucose metabolism in the present study. These findings suggest that the effects of individual dietary SFAs on glucose metabolism may differ. Plasma stearic acid, by contrast, was directly related to 2-h glucose levels in the joint model for male genotype groups (data not shown). This observation is compatible with earlier findings of a direct relationship between stearic acid levels and the risk of T2D (Vessby *et al*. 1994a, Wang *et al*. 2003). However, a significant interaction between this plasma fatty acid and the *PPARG* Pro12Ala genotype on 2-h glucose levels was found (Study I), and the inverse association detected in male *Pro/Pro* homozygotes remained unchanged after further adjustments. An explanation for this observation remains to be elucidated.

Dietary γ-linolenic acid

In men, a significant interaction existed between the *PPARG* Pro12Ala polymorphism and dietary γ-linolenic acid on the 2-h plasma glucose concentration, and an inverse association was observed in the *Pro/Pro* men in the genotype-stratified analyses (Study I). That γ-linolenic acid may have advantageous effects on glucose metabolism has been suggested earlier by experimental studies. Oil rich in γ-linolenic acid decreased serum glucose concentration compared with oil rich in saturated fat or a fat mixture rich in linoleic acid in male rats (Ide *et al*. 2001). The effect was suggested to be due to increased insulin-dependent glucose oxidation and incorporation into lipids in adipocytes. Importantly, PPAR-γ ligands, such as TZDs rosiglitazone and troglitazone, have been shown to increase glucose uptake into adipocytes and incorporation into TGs (Tafuri 1996, Mukherjee *et al*. 2000, Guan *et al*. 2002), which directly increase whole-body glucose disposal (Rangwala and Lazar 2004). In another study in male rats, γ-linolenic acid reduced NEFA concentrations and body fat content, as well as facilitated fatty acid β-oxidation in the liver (Takada *et al*. 1994), which may also enhance glucose metabolism. No explanation for the inverse association in the *Pro/Pro* homozygous but not in the *Ala* allele carrier men has yet emerged. Interestingly, supplementation with oil concentrate rich in γ-linolenic acid was shown to reduce spontaneous food intake, weight gain and adiposity of genetically obese (*fafa*) but not lean (*FaFa*) male Zucker rats (Phinney *et al*. 1993).

The fact that increasing proportions of γ-linolenic acid in serum have been observed according to the deterioration of glucose tolerance (Salomaa *et al*. 1990, Vessby *et al*. 1994a), while dietary γ-linolenic acid has been beneficially associated with glucose metabolism in experimental studies as well as in the present study points to the importance of investigating both dietary and plasma/tissue fatty acids in relation to glucose metabolism.

The present findings concerning γ-linolenic acid furthermore emphasize the importance of investigating the intake of individual fatty acids, even those with absolutely minute intake levels, in addition to (or instead of) fatty acid intake at the group level of SFAs, MUFAs and PUFAs. Small differences in the structure of fatty acids may contribute to large differences in effects, *e.g*., through specific binding to nuclear receptors. Good-quality data on the individual fatty acid content of foods in nutrient databases are a prerequisite for these analyses.

Dietary arachidonic acid

In women, a significant interaction was observed between the *PPARG* Pro12Ala polymorphism and dietary (Study I) as well as plasma (for interaction $P = 0.020$) arachidonic acid on 2-h glucose levels, although in the stratified analyses, the opposite associations in the genotype groups (direct in the *Pro/Pro* homozygous and inverse in the *Ala* allele carriers) did not

reach significance. Based on this finding, it could be argued that one explanation for the earlier discrepant results concerning associations of serum arachidonic acid with glucose metabolism (Pelikánová *et al*. 1989, 2001, Salomaa *et al*. 1990, Vidgren *et al*. 1996; summarized by Lovejoy 1999) could be an interaction of arachidonic acid with the *PPARG* Pro12Ala polymorphism.

The mechanisms which might explain the opposite associations in the genotype groups are at the moment unclear. However, as arachidonic acid has been shown to exert effects that can both enhance and deteriorate glucose metabolism, the finding is reasonable. Arachidonic acid has the potential to downregulate GLUT4 in adipocytes (Tebbey *et al*. 1994), possibly via PPAR-γ (Long and Pekala 1996). On the other hand, arachidonic acid inhibits the expression of resistin (Haugen *et al*. 2005), which has been linked to adipose tissue and liver insulin resistance, and to increased HGP and glucose levels (Wolf 2004). Notably, although arachidonic acid was found to activate PPAR-γ only weakly, arachidonic acid metabolites, like eicosanoids, seem to be more effective (Dussault and Forman 2000).

Dietary and plasma marine *n*–3 fatty acids EPA and DHA and fish intake

A significant interaction between the *PPARG* Pro12Ala polymorphism and plasma cholesterol ester DHA on the fasting serum NEFAs was observed in men (Study II). In the genotype-stratified models, high plasma DHA was inversely associated with NEFA levels only in men homozygous for the *Pro* allele. In the stratified models, also dietary EPA and DHA as well as fish intake showed similar inverse associations with this outcome variable in *Pro/Pro* men. Thus, the present findings suggest beneficial effects of EPA and DHA on NEFA levels in *Pro/Pro* men as well as in all women. The findings are somewhat consistent with the results of Gustafsson *et al*. (1998), according to which supplementation with marine *n*–3 fatty acids reduced serum NEFA concentrations in healthy subjects, although possible modifying effects of genetic factors were not studied.

Beneficial associations between marine *n*–3 fatty acids and insulin resistance and glucose levels were observed mainly in *Ala* allele carriers. A high proportion of plasma EPA was associated with low insulin resistance in *Ala* allele carriers, while no association was seen in *Pro/Pro* genotype carriers (Study II). Furthermore, in women, a high intake of fish was associated with lower 2-h glucose concentration in *Ala* allele carriers, but no such association was seen in *Pro/Pro* genotype carriers (Study II). These observations of marine *n*–3 fatty acids being associated with NEFA levels in *Pro/Pro* men, but with insulin resistance and glucose levels in *Ala* allele carriers suggest that these fatty acids may affect metabolism by different mechanisms in the genotype groups. Moreover, possible beneficial effects on insulin resistance in the *Ala* allele carriers are not necessarily mediated via NEFAs.

Overall, the present results are in accordance with some earlier observations and the prevailing notion that fish consumption and high intake and plasma/tissue concentrations of marine *n*–3 fatty acids may exert beneficial effects on glucose metabolism (Feskens *et al*. 1991, 1995, Adler *et al*. 1994, Baur *et al*. 1998, Ekblond *et al*. 2000, Salmerón *et al*. 2001, Thorsdottir *et al*. 2004; reviewed by Hu *et al*. 2001b, Mann 2002, World Health Organization 2003 and Steyn *et al*. 2004). The present findings did, however, also suggest that the effects may be restricted to *PPARG Ala* allele carriers. These findings may provide one explanation for the discrepancy in earlier results suggesting no independent association (Salomaa *et al*. 1990, Borkman *et al*. 1993, Vessby *et al*. 1994a, b, Marshall *et al*. 1997, van Dam *et al*. 2002, Wang *et al*. 2003, Harding *et al*. 2004) or even a disadvantageous relation (Bjerregaard *et al*. 2000, Mori *et al*. 2000, Dewailly *et al*. 2001a, b) of marine *n*–3 fatty acids with glucose metabolism. Of note, in the KANWU study, a randomized controlled trial, supplementation with long-chain *n*–3 fatty acids had no effect on insulin resistance in the overall population (Vessby *et al*. 2001), and no interaction with the *PPARG* Pro12Ala polymorphism with respect to fasting glucose, insulin or NEFA levels was observed (Lindi *et al*. 2003), probably because of the limited number of subjects (n = 72) included in the latter analysis.

An explanation for the suggested difference in the associations between *n*–3 fatty acids and glucose metabolism between the *PPARG* Pro12Ala genotypes based on the present results is at present unclear. However, one mechanism might be via different effects on the production of leptin, which may interfere with insulin signalling (Auwerx 1999). Reported effects of long-chain *n*–3 fatty acids on leptin have been mixed (Lombardo and Chicco 2006); both increased (Murata *et al*. 2000, Peyron-Caso *et al*. 2002) and decreased (Jang *et al*. 2003) leptin expression and levels have been observed.

Summary

In summary, the findings of the present analyses concerning especially the untoward associations of palmitic acid and the beneficial associations of marine *n*–3 fatty acids with measures of glucose metabolism are in line with the prevailing notion that SFAs may increase and *n*–3 PUFAs decrease the risk of T2D (World Health Organization 2003, Steyn *et al*. 2004). These findings, however, further indicate that these associations may be dependent on the *PPARG* Pro12Ala polymorphism and may possibly be sex-specific. A high intake of palmitic acid may adversely affect fasting glucose levels and insulin sensitivity, especially in *Ala* allele carrier women. The beneficial effects of long-chain marine *n*–3 fatty acids on glucose metabolism may also be most evident in *Ala* allele carriers.

Consistent with the results of Luan *et al*. (2001) and Pisabarro *et al*. (2004), our findings suggest that *Ala* allele carriers may be especially re-

sponsive to dietary fatty acids as regards glucose metabolism. In accordance with this, the Pro12Ala genotype has been shown to be responsive to rosiglitazone treatment (Kang *et al*. 2005). In contrast to these findings, however, Robitaille *et al*. (2003) reported that *Pro/Pro* homozygotes seemed to be sensitive to dietary stimuli. These inconsistencies stress the need to further study the topic.

As reaching unbiased data on dietary intake is difficult, the different associations of dietary fatty acids with glucose metabolism between the genotype groups could also have been due to biased dietary reporting. In this case, it would mean that reporting was biased according to the genotype group. Although this initially seems unlikely, it cannot be totally ruled out. Steinle *et al*. (2002) reported linkage to eating behaviour called disinhibition on chromosomal regions in proximity to genes encoding, among others, for PPAR-γ. Disinhibition describes loss of cognitive avoidance of eating to control body weight, leading to overeating.

As the differences in metabolic events predisposing to or protecting from T2D between the *PPARG* Pro12Ala genotypes are poorly known at present, it is understandable that the mechanisms explaining different associations between fatty acids and different outcome measures of glucose metabolism in these genotype groups are at the moment unclear. The target genes of the PPAR-γ are numerous (Auwerx 1999, Rocchi and Auwerx 1999, Berger and Moller 2002), and therefore the effects of PPAR-γ on glucose metabolism are likely to be complex and also dependent upon the particular ligand, *e.g*., the fatty acid involved. The diversity of the effects of different fatty acids may be due to the subtle structural differences between fatty acids, affecting their affinity for the receptor, inducing differential effects on the coactivator/corepressor binding surface and influencing their potency as ligands (Kliewer *et al*. 1997, Khan and Vanden Heuvel 2003, Sampath and Ntambi 2005). Probably similarly as in the case of TZDs (Berger *et al*. 1996), the anti-diabetic effects of fatty acids may be proportional to their binding affinity for PPAR-γ. In this respect, it is of interest that γ-linolenic acid, which was demonstrated to be one of the fatty acids interacting most efficiently with all PPARs (Xu *et al*. 1999), showed the strongest association with glucose metabolism here.

Trans fatty acids, which may adversely affect glucose metabolism (World Health Organization 2003, Parillo and Riccardi 2004, Steyn *et al*. 2004), had to be excluded from the present analyses because at the time of the gathering of the dietary data manufacturers were reducing the use of partially hydrogenated oils in margarines, and no reliable *trans* fatty acid intake data could be obtained. Furthermore, only the interaction between the *PPARG* Pro12Ala polymorphism and fatty acids was investigated in this study. Although according to the prevailing knowledge, this polymorphism seems to be among the most interesting ones contributing to T2D, obviously there are also other polymorphisms that may have interaction

with fatty acids with respect to glucose metabolism. In fact, associations between fatty acids and glucose metabolism may turn out to be far more complicated than thus far understood.

Plasma surrogates of desaturase enzymes

Previous animal (Park *et al.* 1997) and human studies (Risérus *et al.* 2005) have provided evidence that a genetic component plays a role in determining Δ9-desaturase (or stearoyl-CoA desaturase, SCD) expression. Specifically, heterozygous PPAR-γ deficiency has been shown to reduce expression of Δ9-desaturase (Yamauchi *et al.* 2001). In line with this, a tendency was observed in the present study for a lower Δ9-desaturase activity surrogate in the *Ala* male allele carrier than in *Pro/Pro* men (0.277 *vs.* 0.304, $P = 0.051$). Interaction with a P value of 0.040 between this desaturase and the *PPARG* polymorphism on insulin resistance in men suggests also a role for genetic regulation. Earlier findings concerning the direct associations of plasma palmitoleic acid levels with impaired glucose metabolism (Vessby *et al.* 2002) may thus be partly explained by the genetic regulation of Δ9-desaturase (Kunešová *et al.* 2002).

Several studies have found inverse associations between the indices of Δ5-desaturase activity and insulin resistance and other measures of disordered glucose metabolism (Salomaa *et al.* 1990, Borkman *et al.* 1993, Pan *et al.* 1995). Elevated Δ5-desaturase activity might contribute to improved insulin action by increasing the proportion of long-chain fatty acids in the membranes (Borkman *et al.* 1993). The present results concerning the associations between Δ5-desaturase and glucose metabolism are in line with earlier findings, although the results also suggested that these associations may be dependent on the *PPARG* Pro12Ala polymorphism. The surrogate of Δ5-desaturase activity was beneficially associated with measures of glucose metabolism only in *Ala* allele carriers (Study I). These findings are concordant with the proposal that, in addition to sex, dietary fatty acids and physical activity (Andersson *et al.* 2002, Vessby *et al.* 2002, Burdge *et al.* 2003, Pawlosky *et al.* 2003, Nakamura and Nara 2004), genetic factors may contribute to determine the activity of desaturase enzymes, and thus, body fatty acid composition and its effects on glucose metabolism (Storlien *et al.* 1993, 1996b, Pan *et al.* 1995, Kunešová *et al.* 2002).

An alternative explanation for the observed associations between Δ5-desaturase and glucose metabolism could, however, be foetal programming. In rats, an inverse association between the activity of Δ5-desaturase and insulin resistance was seen in the offspring of a maternal low-protein group, but not in the control group (Ozanne *et al.* 1998). Thus, the relationship between Δ5-desaturase and insulin resistance seems to be determined similarly by early nutritional deprivation and the Pro12Ala polymorphism of *PPARG*. Another similarity between the nutritionally deprived animals and the *Ala* allele carriers is a difference in the fatty acid composition com-

pared with control animals or the *Pro/Pro* homozygotes. The level of docosapentaenoic acid (22:5*n*–3), an elongation product of EPA, tended to be higher in the phospholipids from the low-protein animals than in control animals, while the plasma level of EPA was higher in the *Ala* allele carrier women than in the *Pro/Pro* women (plasma levels of docosapentaenoic acid were not determined) (Study II).

Based on the present findings and earlier observations in rats, it is tempting to suggest that the *Ala* variant of *PPARG* and deprived early nutrition could lead to a similar 'metabolic milieu', and thus, to respective programming of relationships between fatty acids and glucose metabolism. In addition, the thrifty genotype may contribute to the thrifty phenotype. Support for the view that foetal programming could also be genetically determined and involve the *PPARG* Pro12Ala polymorphism comes from a study showing a direct association between reduced growth and disordered insulin metabolism in carriers of the *Pro/Pro* genotype, but not in *Ala* allele carriers (Eriksson *et al.* 2002). Interestingly, in one study in rats, fish oil supplementation prevented the development of abnormal glucose metabolism in adult offspring of rats fed a low-protein diet (Joshi *et al.* 2003). The authors suggested that marine *n*–3 fatty acids could thus contribute to the 'metabolic programming' of foetal adaptation to maternal undernutrition (Joshi *et al.* 2003). Similarly, if the endogenous production of EPA is higher in *Ala* allele carriers, as suggested by the present study, this might protect this genotype from the deterioration of glucose metabolism.

6.2.2 Dietary and plasma tocopherols and carotenoids

Tocopherols

An inverse association between the dietary intake of α-tocopherol and the fasting plasma glucose concentration was observed in women (Study III). As α-tocopherol may play a role in the maintenance of endothelial function (Laight *et al.* 2000, Wu *et al.* 2005), the impairment of which characterizes subjects with IFG (Vehkavaara *et al.* 1999), it could be speculated that the observed advantageous association between dietary α-tocopherol and the fasting plasma glucose might be mediated via enhanced endothelial function.

Earlier, one prospective study found an inverse association between the dietary intake of γ-tocopherol and the risk of T2D (Montonen *et al.* 2004), while two cross-sectional studies reported a direct relation between plasma γ-tocopherol concentration and GHb (Bates *et al.* 2004, Ford *et al.* 2005). In our analyses, the direct relation between plasma γ-tocopherol and insulin resistance in men was abolished after adjustment for dietary covariates (Study III). Although the role of γ-tocopherol in

glucose metabolism remains unsettled, the above observations suggest that associations of dietary *vs.* plasma γ-tocopherol levels with glucose metabolism may differ and both should be included in future studies.

An explanation for the direct association between plasma α-tocopherol and 2-h plasma glucose levels observed in both sexes (Study III) continues to elude us. The finding is, however, in line with earlier reports of increased vitamin E levels in subjects with diabetes (Vatassery *et al.* 1983, Caye-Vaugien *et al.* 1990, Krempf *et al.* 1991, Thompson and Godin 1995). In their study of 35 subjects with T2D, Krempf *et al.* (1991) noted a direct, although non-significant ($P = 0.07$) relation between vitamin E concentration and GHb. Therefore, as supplementation with vitamin E has not increased glucose levels in previous studies (Meydani *et al.* 1994, 1998), and as the direct relation between plasma α-tocopherol and the 2-h plasma glucose remained significant after adjustment for serum cholesterol concentration in the present study, it is tempting to speculate that the observed association, instead of indicating that a high intake of α-tocopherol adversely affects glucose levels, may suggest increased α-tocopherol levels being due to underlying metabolic alterations in glucose metabolism (manifested as increased post-load glucose levels) in subjects predisposed to T2D. In an animal model of type 1 diabetes, vitamin E levels were increased in both untreated diabetic rats and in asymptomatic diabetes-prone animals (Behrens *et al.* 1984). Since insulin treatment restored elevated vitamin E levels in the diabetic animals, the raised tocopherol concentrations seem to have been due to the diabetic state. The authors suggested that the utilization of vitamin E might be impaired in genetically predisposed individuals (Behrens *et al.* 1984). Whether that interpretation has any relevance to explain the present finding, and whether the mechanism could be related to the prevention of vitamin E binding to cell membranes due to glycation or oxidative modification of membrane proteins (Kunisaki *et al.* 1993, Traber and Packer 1995) warrants further attention.

Carotenoids

The inverse associations observed between the intake of carotenoids and the fasting plasma glucose concentration as well as between plasma β-carotene and insulin resistance in men (Study III) are in line with earlier studies suggesting beneficial associations between carotenoids and glucose metabolism (Ford *et al.* 1999, Facchini *et al.* 2000, Coyne *et al.* 2005, Czernichov *et al.* 2006). The reason for the lack of respective associations in women of the present study is unknown. Nevertheless, Wang *et al.* (2006) found no association between lycopene intake and the risk of T2D in women. In their study, however, the correlation between dietary and plasma lycopene was weak ($r = 0.14$), and the lack of an association may also have been due to the uncertainty of measuring lycopene intake.

An explanation for the direct association between plasma β-carotene and the fasting plasma glucose concentration in women (Study III) is also unclear. Some older studies have found increased carotene levels in subjects with diabetes (summarized by Abahusain *et al*. 1999), and thus, it could be argued that the present observation might be explained by increased carotenoid levels due to altered pre-diabetic metabolism.

To summarize, although the present cross-sectional findings suggest that tocopherols and carotenoids might exert some beneficial effects on glucose metabolism, recently reported intervention studies do not support an independent role for vitamin E supplementation (Czernichov *et al*. 2006, Liu *et al*. 2006) or β-carotene supplementation (Liu *et al*. 1999) in the prevention of T2D. Whether some untoward associations between plasma levels of antioxidants and glucose metabolism observed in the present study are due to pre-diabetic metabolism deserves attention. Regarding antioxidants and gene expression, as the chemical structure of vitamin E resembles that of TZDs, which are potent activators of PPAR-γ (Berger *et al*. 2005), some of the effects of vitamin E on glucose metabolism might be mediated via PPAR-γ activation. In addition, some evidence suggests that lycopene may affect the expression of PPAR-γ (Zaripheh *et al*. 2006). Therefore, an interaction between the *PPARG* Pro12Ala polymorphism and the intake of vitamin E and carotenoids on glucose metabolism should be explored in future studies.

6.2.3 Dietary fibre

The finding of an inverse association between dietary fibre intake and insulin resistance (Study IV) is in line with the prevailing notion that dietary fibre may beneficially affect insulin sensitivity (Bessesen 2001). Of note, in our study, both soluble and insoluble fibre was associated with better insulin sensitivity. On the other hand, while the intake of (insoluble) fibre has recently been associated with reduced risk of T2D in several prospective cohort studies (Salmerón *et al*. 1997a, b, Stevens *et al*. 2002, Montonen *et al*. 2003, Schulze *et al*. 2004; reviewed by Parillo and Riccardi 2004, and Montonen 2005), and also in the Finnish Diabetes Prevention Study (Lindström 2006), dietary fibre did not show independent associations with glucose levels in the present study. This might be explained by the majority of the subjects (~ 80%) having NGT. Associations of dietary fibre with glucose levels may become evident only after deterioration of insulin secretion in subsequent stages of the disease.

The mechanisms of the observed associations of dietary fibre with insulin sensitivity cannot be inferred from the present study. Nevertheless, because adjustment for NEFA levels did not abolish the associations, dietary fibre may affect insulin sensitivity also independently of NEFA release (McKeown and Mayer 2004).

As regards the effects of carbohydrates on glucose metabolism, the concepts of glycaemic index and glycaemic load have recently received much

attention (Augustin *et al.* 2002, Jenkins *et al.* 2002, Brand-Miller 2003). The glycaemic index describes the difference in blood glucose response after ingestion of carbohydrates from different foods (Arvidsson-Lenner *et al.* 2004) and can be used to categorize carbohydrate foods as those rapidly absorbed and those more slowly absorbed. A high glycaemic index and a high glycaemic load, which is the product of the glycaemic index and the quantity of the carbohydrate in a food, have been associated with impaired glucose metabolism (Salmerón *et al.* 1997a, b, Hodge *et al.* 2004, Schulze *et al.* 2004). Their use in the guidance of food choices is, however, limited because they refer to glycaemic carbohydrates and not to food (Monro 2003). They are therefore not suitable for labelling of foods (Arvidsson-Lenner *et al.* 2004). On the other hand, the concept of dietary fibre is familiar to lay people, and information on the fibre content of foods already exists on many packages. These practical issues combined with the prevailing fairly strong evidence of beneficial effects of dietary fibre on glucose metabolism urge further use of the concept of dietary fibre and the promotion of fibre intake to prevent T2D (Bessesen 2001).

6.3 Strengths and limitations of the epidemiological approach used

Epidemiological studies can be regarded as 'experiments of nature of which the epidemiologist is a passive observer' (Clayton and Hills 1993). By exploring associations between exposure and outcome variables without *a priori* assumptions and often also without knowledge about possible mechanisms, thus far undetected associations can be found and new hypotheses provided (Davey Smith 2001, Sempos and Looker 2001). One of the obvious strengths of nutritional epidemiology is the study of associations between outcomes and realistic levels of dietary exposure as habitual dietary intake is measured. In animal studies, the experimental doses of exposure factors are often very high compared with those of habitual intake in man, and extrapolations from animal studies to humans must be done with caution. Another major strength of epidemiological studies is exploring these associations in man. This eliminates doubt about the generalizability of results because of possible species-specific differences in metabolism (O'Rahilly *et al.* 2005). Epidemiological studies, in addition to randomized controlled trials, thus serve to explore whether findings of *in vitro* and animal studies are observed with relevant exposure levels in the human population.

Imprecision in the measurement of exposures, outcomes and confounders, as well as potentially inadequate adjustment for unknown or unquantifiable covariates are the main limitations of epidemiological studies. Possible measurement errors of the dietary data in the present study were

discussed in Section 6.1. The confounders used in the regression models were explained in the original papers. They included several demographic, clinical and dietary factors known or assumed to be relevant confounders or mediators of the associations under investigation. Some obvious confounding variables, such as age, were adjusted for, although they were not necessarily significant in all models. The choice of confounders is, however, not straightforward because prevailing knowledge of possible confounders may be limited or inconsistent. Smoking, for example, has been associated with blood carotenoid and vitamin E concentrations in some although not all studies (Rock *et al*. 1996, Clinton 1998, Ford and Sowell 1999, Rao and Agarwal 1999, Jandacek 2000). Thus, although in the present analyses regression models were adjusted for many potential confounders, inadequate control for incompletely measured, unmeasured or unknown confounding factors that might explain some of the relationships cannot be ruled out.

As regards adjustment for anthropometric confounders, it could be argued that inclusion of both BMI and WHR in a model is not appropriate because of their high mutual correlation. However, as BMI and WHR measure different aspects of body fatness, and as both overall obesity and abdominal obesity have been associated with diminished glucose disposal (Farin *et al*. 2006) and with increased risk of T2D (Wang *et al*. 2005), they could be introduced simultaneously to the models.

Avoiding overadjustment of the models is also an issue in statistical analyses of epidemiological studies. The true association between a dietary exposure and an outcome may be obscured if the model is controlled for covariates that might be on the causal pathway. For example, if obesity is an intermediary between dietary fat and insulin resistance, adjustment for BMI could underestimate the contribution of dietary fat. As dietary fatty acid variables in the present study remained related to measures of glucose metabolism after accounting for BMI, adjustment for this covariate was, however, reasonable and indicates that dietary fat may affect glucose metabolism also independently of obesity.

The size of our study population was rather small for investigating an interaction between diet and genes. Therefore, the possibility of false-negative results (Type 2 error) cannot be excluded. However, the number of participants was comparable with that in the study of Luan *et al*. (2001), which found an interaction between the same polymorphism and the ratio of dietary PUFAs to SFAs on BMI and fasting insulin levels. In addition, because of multiple testing of associations between several exposure and outcome variables, false-positive results (Type 1 error) cannot be excluded. Regarding regression models studying interaction between fatty acids and the Pro12Ala polymorphism on glucose metabolism in Study I, the P value was restricted to be significant only when < 0.01, which somewhat reduced the possibility of false-positive results. However, it remains possible that the observed associations occurred by chance. This nevertheless seems un-

likely for the findings that agree with prior evidence from experimental research, and for which a possible mechanism of action has been presented. Overall, congruence across the results of epidemiological and experimental studies supports the credibility of the findings.

7 Summary and Conclusions

T2D is a disease affecting genetically predisposed individuals with contributing lifestyle factors. Compared with ancient times, current intakes of *n*–3 fatty acids, antioxidative nutrients and fibre are low and may be poorly compatible with the ancient genetic constitution of man. This discrepancy may contribute to the development of multifactorial diseases such as T2D (Arab 2003, Simopoulos 2003, Cordain *et al.* 2005).

Recent studies have shown that T2D may be largely preventable by lifestyle intervention (Pan *et al.* 1997b, Tuomilehto *et al.* 2001, Knowler *et al.* 2002), but it is not yet known whether efficient dietary modifications differ according to the genetic susceptibility of subjects. Therefore, in the present study, associations between dietary factors and measures of glucose metabolism predicting T2D were examined in a high-risk population, *i.e.*, in non-diabetic relatives of patients with the disease. The dietary factors investigated included dietary and plasma cholesterol ester fatty acids, carotenoids and tocopherols as well as dietary fibre. Interactions between the *PPARG* Pro12Ala polymorphism and fatty acids on glucose metabolism were also explored. This common polymorphism is assumed to be of practical significance, as it plays an important role in the regulation of glucose metabolism (Hansen and Pedersen 2005).

The dietary study was part of the follow-up investigation of the Botnia Study, which is a prospective family study of genetic and metabolic defects of T2D (Groop *et al.* 1996). The study was approved by the local ethics committees. Inclusion criteria of the dietary study were as follows: 1) a first- or second-degree family history of T2D, 2) an age of 20-70 years at the time of the Botnia baseline investigation and 3) NGT or IGT according to the 1985 WHO criteria (World Health Organization 1985). A consecutive sample of 746 subjects was invited to participate, 603 (81%) of whom agreed.

Dietary intake was measured by means of two 3-day estimated food records spaced on average 6 months apart. In Studies I, II and III, dietary data were determined from the first 3-day period, and in Study IV, from both 3-day food records. The field work of the dietary study was carried out by local research staff after completing an intensive training programme. Each 3-day recording period included two weekdays and one day of the weekend. The amount of food and drink was estimated using a booklet of food photographs with different portion sizes (Haapa *et al.* 1985) or with household measurement units. After the recording period, the food records were checked together with the participant by research staff. The

food intake data were analysed using the Finnish National Food Composition Database (Ovaskainen *et al*. 1996).

The medical screening included measurements of weight, height, waist and hip circumferences, blood pressure, and samples for serum lipids and for plasma cholesterol ester fatty acids, carotenoids and tocopherols. Standardized OGTT and IVGTT were performed. Data on smoking, education and physical activity were obtained by a structured questionnaire.

Associations of dietary and plasma exposure variables with measures of glucose metabolism were studied with multiple linear regression analysis. The main outcome variables were HOMA insulin resistance, β-cell function (insulinogenic index, first-phase insulin secretion during IVGTT), and fasting and 2-h post-load (OGTT) plasma glucose and serum NEFA concentrations. As the participants could be relatives, the analyses were performed with the PROC MIXED procedure (SAS/STAT software; SAS Institute Inc., Cary, NC), where the family connection was considered. Potential interaction with the *PPARG* Pro12Ala polymorphism in Studies I and II was explored by models including the main effects and the product term of the genotype and the exposure variable, and thereafter by performing genotype-stratified analyses. To evaluate whether associations between exposure and outcome variables were independent of potential confounding factors, these were added to the models. Potential confounders were identified from earlier reports and from correlation analyses of the present studies.

The major findings of Studies I to IV can be summarized as follows:

Study I

In men, regression models studying interaction between the *PPARG* Pro12Ala polymorphism and dietary and plasma fatty acids showed interaction for the intake of γ-linolenic acid and for plasma stearic acid on the 2-h plasma glucose concentration. In the genotype-stratified analyses, high intake of γ-linolenic acid and high plasma stearic acid proportion were associated with low 2-h plasma glucose in *Pro/Pro* men, whereas no association was seen in *Ala* allele carriers.

In women, an interaction between the *PPARG* Pro12Ala polymorphism and dietary palmitic acid on the fasting plasma glucose concentration was observed. In the stratified analyses, palmitic acid was directly related to the outcome variable in *Ala* allele carriers, but not in *Pro/Pro* women.

In women, an interaction was also observed between the *PPARG* Pro12Ala polymorphism and dietary arachidonic acid on 2-h glucose levels, although in the stratified analyses, the opposite associations in the genotype groups (direct in *Pro/Pro* homozygous and inverse in *Ala* allele carriers) did not reach significance.

Furthermore, in women, there was an interaction between the *PPARG* Pro12Ala polymorphism and Δ5-desaturase on insulin resistance and on

the 2-h plasma glucose concentration; inverse associations were observed in *Ala* allele carriers, but not in *Pro/Pro* homozygotes.

In addition to the above-mentioned genotype-specific associations, high plasma dihomo-γ-linolenic acid was associated with high insulin resistance and with high 2-h plasma glucose concentration in the combined group of women.

Study II

In men, an interaction was present between the *PPARG* Pro12Ala polymorphism and plasma DHA on the fasting NEFA concentration, and genotype-stratified models showed an inverse association in *Pro/Pro* homozygotes, but not in *Ala* allele carriers. In the stratified models, also the intakes of fish, EPA and DHA showed inverse associations with this outcome variable in *Pro/Pro* men, but not in *Ala* allele carriers.

An interaction between the *PPARG* Pro12Ala polymorphism and plasma EPA on insulin resistance was observed in men (*P* for interaction in women 0.12). When men and women were analysed together in the stratified model, a high proportion of plasma EPA was associated with low insulin resistance in *Ala* allele carriers, while no association was seen in *Pro/Pro* genotype carriers.

An interaction between the *PPARG* Pro12Ala polymorphism and fish intake on the 2-h glucose concentration was found in women. Genotype-stratified models showed an inverse association in *Ala* allele carriers, but not in *Pro/Pro* homozygotes.

Finally, the proportion of plasma EPA was higher in *Ala* allele carrier women than in female *Pro/Pro* homozygotes.

Study III

The dietary intake of α-tocopherol was inversely associated with the fasting plasma glucose concentration in women. By contrast, a direct association between plasma α-tocopherol concentration and 2-h plasma glucose levels was observed in both men and women.

An inverse association between the intakes of α-carotene, β-carotene and lycopene and the fasting plasma glucose concentration was observed in men. In addition, plasma β-carotene was inversely related to insulin resistance in men. In women, on the other hand, plasma β-carotene showed a direct association with the fasting plasma glucose.

Study IV

The intakes of total, insoluble and soluble fibre were inversely related to insulin resistance in men and women combined.

When interpreting the present findings, it should be kept in mind that they pertain to subjects with a family history of T2D and may not be generalizable beyond the study population. Because of the observational cross-sectional nature of the study, inferences about the cause and effect cannot be made. The associations found require confirmation in long-term prospective studies, and our hypotheses should be tested in randomized controlled trials.

The results concerning associations between dietary fibre and insulin resistance are consistent with the prevailing recommendations urging increased intake of fibre to prevent T2D (Bessesen 2001, World Health Organization 2003, Parillo and Riccardi 2004, Steyn *et al*. 2004). Beneficial associations observed between the intake of carotenoids and glucose levels stress that a high consumption of vegetables, fruits and berries rich in carotenoids might also play a role in the prevention of T2D. Whether tocopherols have an independent beneficial association with glucose metabolism is questionable. The present findings were somewhat equivocal, suggesting a beneficial association for dietary α-tocopherol with fasting glucose levels, but a deleterious association for plasma α-tocopherol with 2-h glucose levels. Importantly, recently reported intervention studies do not support an independent role for vitamin E in the prevention of T2D (Czernichov *et al*. 2006, Liu *et al*. 2006). However, interactions between antioxidative nutrients and genetic factors, including the *PPARG* Pro12Ala polymorphism, on glucose metabolism should be explored in future studies.

Interactions observed between the *PPARG* Pro12Ala polymorphism and fatty acids with respect to glucose metabolism imply that genetic polymorphisms may change the metabolic response to diet, thus influencing the risk patterns of the disease. Seemingly contradictory findings of previous research may be partly explained by genetic heterogeneity within and between study populations. Although T2D has been assumed for a long time to develop as a result of an interaction between genetic and environmental factors, including diet, information to support this assumption has just started to become available.

Based on the present findings, it can be argued that a high intake of the main dietary SFA, palmitic acid, may be adversely associated with high fasting glucose levels mainly in female *Ala* allele carriers. Furthermore, it can be hypothesized that the *PPARG* Pro12Ala polymorphism may modify the metabolic response to dietary marine fat. The beneficial associations of high fish consumption and high intake of marine *n*–3 fatty acids with insulin resistance and glucose levels may be restricted to (female) carriers of the *Ala* allele. In addition, the conversion of α-linolenic acid to EPA may be higher in female *Ala* allele carriers than in *Pro/Pro* homozygous women. A higher conversion rate possibly during the foetal period and in adult life might contribute to enhanced insulin action in *Ala* allele carriers.

Among the practical implications of these results is that in future studies on diet and glucose metabolism, genetic factors, such as the *PPARG* Pro12Ala polymorphism, should be taken into account. Inclusion of genetic factors in the dietary analyses may provide clues about the role of genetic *vs*. dietary factors in determining, *e.g*., the metabolism of fatty acids. At present, it is unknown to what extent the plasma/tissue fatty acid levels and their relationships to glucose metabolism (*e.g*., those of dihomo-γ-linolenic acid) are determined by dietary and other lifestyle factors and to what extent by genetic factors or by the underlying pathogenic process. Furthermore, in future studies, it is of the utmost importance to explore associations of both dietary and plasma/tissue levels of nutrients in relation to glucose metabolism, as differences may exist between these (*e.g*., the associations of dietary *vs*. serum γ-linolenic acid with glucose metabolism may differ). The molecular mechanisms behind the observed associations also require investigation.

If the present observations concerning interactions between the *PPARG* Pro12Ala polymorphism and fatty acids on glucose metabolism are replicated in other populations and with other study designs, the findings have important implications for the prevention of T2D. If associations of fatty acids with glucose metabolism are modulated by the genetic makeup of an individual, by taking this into account the prevention of the disease may be enhanced. However, much additional research is needed to elucidate the role of diet in the aetiology of T2D. The study of numerous nutrient-gene interactions is warranted. Clearly, the last chapter of the topic 'diet in the aetiology of T2D' has not yet been written.

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